TARGET ORGAN TOXICITY:
IMMUNE SYSTEM

ENVIRONMENTAL HEALTH PERSPECTIVES

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service–National Institutes of Health
National Institute of Environmental Health Sciences

VOLUME 43, February 1982

SUBSCRIPTION INFORMATION

Subscription rates are as follows: Single copies $7.00, foreign $8.75, domestic $41.00 per year, foreign $51.25 per year. The yearly subscription is for 6 regular issues. Please send check or money order, in advance to the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

The Secretary of Health and Human Services has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1983.

ENVIRONMENTAL HEALTH PERSPECTIVES is a publication of the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services. Opinions expressed herein, however, are not necessarily endorsed nor shared by any of the above agencies.

DHHS publication No. (NIH) 82-218

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Print Schedule: Volume 44 will present the proceedings from the Symposium on Target Organ Toxicity: Eye, Ear and Other Special Senses

Volume 43, February 1982

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Target Organ Toxicity: Immune System

Co-Sponsored by
Society of Toxicology
and
National Institute of Environmental Health Sciences

Arlington, Virginia
October 20-21, 1980

Environmental Health Perspectives
Vol. 43, pp. 1-2, 1982

Opening Remarks and Objectives

by Albert E. Munson* and Jack H. Dean†

This symposium is the eleventh in a series of Target Organ Toxicity meetings co-sponsored by the National Institute of Environmental Health Sciences and the Society of Toxicology. These meetings are coordinated by Drs. Robert Dixon, Joseph Borzelleca and Perry Gehring, who recognize the need for period review of methods used in the assessment of chemically induced toxicity.

The functions of the immune system are defense, homeostasis and surveillance. Stimuli can be either endogenous or exogenous, and the immune response either specific or nonspecific. This conference was organized in response to the increasing interest in the immune system as a target organ for toxicity.

The interactions of environmental chemicals or drugs with the immune system may result in three types of undesirable effects: (1) those determined by immunodeficiency or immune suppression, (2) those determined by alterations of host defense mechanisms, and (3) those determined by allergy or hypersensitivity. Recently some drugs have been developed which stimulate immune responses in a beneficial manner. Although the system is remarkably resilient, drugs, chemicals, ionizing radiation and malnutrition can impair its function. There is increasing evidence that chronic, subclinical exposure to certain chemicals may depress immune responsiveness and increase susceptibility to infectious agents and cancer.

A vast array of cell types and cell products are induced to interact with a diversity of responses when the immune system is challenged by antigenic substances. The sophisticated cellular and molecular interactions operant in the immunologic response have attracted the attention of both basic as well as applied scientists. Attention has been focused on the thymus-derived lymphocytes (T-cells), the bursa equivalent-derived lymphocytes (B-cells) and the macrophage. The T-lymphocytes are involved in cell-mediated immunity, B-lymphocytes in humoral immunity, and macrophages with persistent intracellular organisms. A number of new laboratory approaches are currently being developed and validated to define alterations in immune parameters and host resistance following chemical exposure.

The focus of this conference is immunotoxicology. The meeting was structured to review the structure and function of the immune system; describe specific *in vitro* and *in vivo* methods for assessing cellular and humoral immunity; present whole animal models for monitoring host resistance; introduce newer approaches for identifying environmental agents which affect immune function; and identify the immune system as a tool in human risk assessment following chemical exposure.

These immune system symposium topics were carefully selected to achieve a comprehensive overview of interest to toxicologists and to clarify the complexities of effector mechanism that mediate immunologic responses and describe their perturbation by chemicals. The goals of the symposium were to increase the understanding of: the structure, function and probable cellular sites where chemicals can alter the immune response; the methods of measuring the functional activity of the humoral and cell-mediated arms of the immune system; the approaches to measuring host resistance to infectious agents and tumors; new strategies for measuring the effects of chemicals on the immune system; and the importance of immunotoxicity to risk assessment.

An excellent group of scientists presented the latest information on the immune system and its importance in toxicity assessment at this symposium. The first session (Session I) dealt with the structure and function of the immune system.

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Session II, chaired by Dr. Jack Dean, concerned methods and approaches for assessing immunotoxicity. Session III, chaired by Dr. S. Gaylen Bradley, dealt with models for studying alterations in host-parasite parameters. Session IV, chaired by Dr. Douglas Archer, provided insight into new approaches in immunotoxicological assessment. A panel discussion by a group of distinguished toxicologists on immunotoxicology and its place in toxicology concluded the symposium.

Several factors will determine the success of evaluating the immune system as part of an overall toxicological assessment. Some cross education of the immunologist and the toxicologist in the other's disciplines is of prime importance. This education must be at both the basic and applied levels. For example, the immunologist should understand the problems of using inbred mice in predicting toxicity, while the toxicologist should understand the difficulty of assessing the immune response in rats. The immunologist needs to recognize that the toxicologist is expected to explain alterations produced by a chemical on an animal's function and the significance of that alteration as well as its extrapolation to man. The toxicologist must recognize that functional immunological assays may prove more sensitive indicators of adverse effects and injury than morphological analyses. The toxicological principles of dose response, time course and reversibility become relevant for the immunologist, while the complexities of the immune response will present new awareness for the toxicologist schooled in those principles.

As the study of the immune system as a target for toxicity is a relatively new area of toxicology, new approaches are needed. Development of new approaches may sometimes involve breaking certain toxicological "commandments," such as not introducing foreign substances into the test animals or using only rats, guinea pigs, dogs and monkeys in toxicological studies. Immunological response to an antigen can serve as a valuable index of immune system toxicity; initial use of an animal whose immune system is well described (the mouse) and for which better diagnostic reagents are available rather than the species normally used in toxicological assessment can also be advantageous.

For the immunotoxicologist to evaluate the effects of a chemical upon the immune system, the consequences of inhibition or stimulation of this system should be considered. Picture an array of responses produced by the chemical by either immunosuppression or immunoenhancement. Increased susceptibility to infection may result from immunosuppression, while a vast continuum of allergic responses and autoimmune diseases could result from immunoenhancement. Integrate into this continuum factors which must be considered by the toxicologist, such as chemical distribution, dose, length, and route of administration, and one begins to see the problems that the immunotoxicologist faces.

Four papers follow in this section, each of which addresses a different aspect of the structure and function of the immune system. In the first paper, Dr. Peter Bick presents an overview of the immune system as a target for toxicity. This is followed by a paper by Dr. Eugene Heise on the diseases associated with immune suppression. Dr. Lucy Rasmussen further elaborates on the consequences of immune suppression, drawing information from drugs known to suppress this system. A major concern of toxicologists for years has been the allergic potential of chemicals; Dr. William Jordan addresses this issue.

Environmental Health Perspectives
Vol. 43, pp. 3-7, 1982

Immune System as a Target Organ for Toxicity

by Peter H. Bick*

Recently, interest has centered on the immune system as a target organ for toxic effects. This seems a reasonable choice, since it can be argued that alterations induced in this system as a result of a toxic insult could lead to impaired immunity. Such an alteration may be manifest in altered disease susceptibility. Documenting toxic effects upon the immune system is a difficult task due to the multifaceted network of specialized cells that carry out immune functions. Because of this complexity, it is necessary to develop a panel of immunologic assays to adequately cover the diverse functions that may be affected by a toxic substance. In the interest of efficiency and cost, it is preferable to organize the assay systems into a tier structure to generate the most information in the shortest time. An overview of the immune system is presented, and an example of one possible tier system of assays is described.

Introduction

As a target organ for toxicity the immune system is, at first glance, a logical choice to investigate. One could reason that prolonged exposure to a substance of low toxicity or an acute exposure to a highly toxic chemical may alter immunological functions and predispose an individual to increased disease susceptibility. In practice, however, documenting such effects is not the easy task one would hope for. This is due to the nature of the immune system, which comprises a large network of organs and varied cell types that carry out interrelated functions. Many of the cellular subpopulations are highly specialized, and there exist numerous avenues of communication and a complex regulatory network that governs responsiveness (*1*). Of selective advantage to the host is the fact that the immune system offers several pathways for handling a challenge. This fact alone complicates toxicologic studies by allowing the possibility of a protective immune response to coincide with a toxic effect. A component of one pathway may be compromised by a toxic insult, yet the host's ability to survive a challenge by an infectious agent may be unchanged. Immunotoxicological studies therefore must be multifaceted to detect such situations.

It is desirable to establish a set of immunologic tests that could be applied to the study of the toxicological properties of a wide range of suspected substances. Such a battery of tests should have the following characteristics: (a) a standardized test panel should be based on sound cellular immunologic techniques; (b) the test panel should be as sensitive and reproducible as possible to facilitate data comparison between laboratories; and (c) the test systems should be as automated as possible to allow sufficient data to be generated from a statistically large sample population. The limitations of each test in relation to what is learned concerning the function of the immune system as a whole should be considered. In order to reach this goal, one must have an overall appreciation of the complexity of the immune system.

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Development, Structure and Function of the Immune System

Cells of the immune system originate from a pluripotent stem cell pool found in the yolk sac and fetal liver during fetal development and in the bone marrow and spleen of the adult (*2-4*). As shown in Figure 1, stem cells may follow one of several lines of maturation. Influential in the development of one major lymphocyte class is the thymus, which originates from the third and fourth pharyngeal pouches at 9 days of gestation in mice, (9 weeks in man). As stem cells pass through and are influenced by the thymus, they mature and acquire the characteristics of thymus-derived T-lymphocytes on their way into the peripheral lymphoid organs. Mature T-lymphocytes seed the thymic-dependent areas of the spleen and lymph nodes and comprise 55-74% of the peripheral blood lymphocytes (*5*, *6*). These cells can be distinguished from other lymphocytes by genetically defined alloantigenic cell surface markers: Thy 1 (theta) and Lyt 1, 2, and 3 (in mice), among others (*7-9*). Such cell surface markers provide handles with which to identify functional subpopulations of T-cells in normal and disease states. T-lymphocytes also respond to the mitogens phytohemagglutinin (PHA) and concanavalin A (con A), which can be used as a gross probe for the integrity of T-cell function (*10*, *11*). T-cells carry out functions of immune regulation by participating as amplifying "helper" cells and "suppressor" cells active in antibody responses to thymic-dependent antigens, mixed lymphocyte responses, delayed-type hypersensitivity (DTH) reactions and the activation of cytotoxic effector T-cells. Effector T-cells participate directly as killer cells against virally infected host cells, malignant cells and foreign tissue, function in DTH reactions and elaborate soluble lymphokine mediators capable of modulating inflammation. T-cells are long-lived lymphocytes, and the thymus atrophies in the adult animal. Some estimates of longevity place some T-cells as existing for 15-20 years. Since there is little replacement of T-cells, alterations in this compartment may be very detrimental. Lesions in the T-cell system result in enhanced susceptibility to viral, fungal and parasitic infections and alter the levels of most antibody responses. Depletion of the thymus gland or stem cell pool during fetal development decimates this branch of the immune response as demonstrated by patients with DiGeorge syndrome. Alterations in discrete T-cell subsets may alter immune responses in a selective manner. For example, patients with systemic lupus erythematosis have below normal suppressor T-cell function, resulting in unregulated antibody production (*12-16*). Evaluation of T-cell function should include test parameters that detect functional activity of the various T-cell subpopulations.

The second major route of lymphocytes differentiation is well defined in avian species. Here the Bursa of Fabricius processes stem cells to become mature B-lymphocytes. No such organ can be defined in mammals, and several sites for B-lymphocyte maturation have been suggested, including bone marrow, spleen and the gut-associated lymphoid tissue. Lymphocytes that follow this pathway become mature B-lymphocytes that possess specific immunoglobulin receptors on the cell surface that are of the same specificity as the antibody to be produced by the cell following activation. The high density of cell surface immunoglobulin (sIg) is a central characteristic that separates B- from T-lymphocytes. As B-lymphocytes mature from stem cells, a number of cell surface changes occur. B-lymphocytes progress from a "pre-B" cell with no surface immunoglobulin to an immature sIgM-bearing cell. Subsequent differentiative steps see the B-cell sequentially acquiring receptors for the Fc portion of IgG, cell surface IgD, immune-response associated (Ia) antigens coded for by the major histocompatibility complex and receptors for complement components (*17-21*). B-lymphocytes with differing cell surface phenotypes represent functionally different subpopulations that respond differentially to tolerance induction and antigenic and mitogenic stimulation (*22-24*). The cell surface markers can be used to delineate and identify alterations in

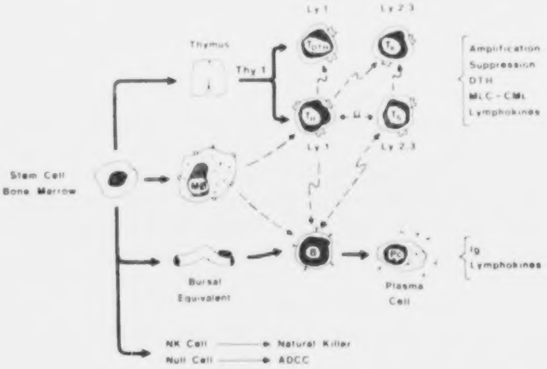

FIGURE 1. Development and organization of the cellular immune system

subpopulations in normal and experimentally modified B-cell populations. Antisera specific for many of the cell surface markers (sIg, Ia) are commercially available, some as hybridoma antibodies, allowing widespread application of phenotype determination and cell depletion. Mature B-lymphocytes are found in the thymic-independent areas of spleen and lymph nodes and represent approximately 25-45% of peripheral blood lymphocytes.

Following activation, B-lymphocytes proliferate and/or differentiate into antibody-producing cells and plasma cells. As this process proceeds, many cell surface markers are lost, and the end-stage plasma cell expresses a new antigen (plasma cell antigen, Pca) and produces quantities of specific immunoglobulin. In contrast to the long-lived T-cell, B-cells turn over frequently and are replaced by newly differentiating stem cells. Alterations in the B-cell compartment may be reversible following the differentiation of new adult B-cells. Toxic insults that result in depletion of B-cell function can result in lowered immunoglobulin levels and increased susceptibility to bacterial infection.

Other lymphocytic cells active in immune cytotoxic functions are the natural killer (NK) cell and the "null" cell active in antibody-dependent cell-mediated cytotoxicity (ADCC). Natural killer cells have cytotoxic potential for certain malignant cells in the absence of prior sensitization. These cells probably represent pre-T cells but do not exhibit traditional T-cell phenotypes (*25-27*). The null cells active in ADCC do not bear traditional B-cell markers but possess receptors for IgG Fc and some T-cell markers and are capable of killing antibody-coated target cells (*28*). The differentiation pathways of these cells are not as well defined s are those of the T and B lymphocytes. The observation that cells active in ADCC bear some T-cell markers suggests that these cells evolve rom the T-cell lineage and that the ADCC and NK effector cell populations may overlap (*29-31*). Although there exist mouse strains that are deficient in NK activity [Beige mice; C57BL/6J-bg (*32, 33*)], is not clear what the long-range effects of efects in ADCC or NK activity may be. These unctions may be compensated for by T-lymphocyte unctions.

The pluripotent stem cell pool also contains the recursors of monocyte-macrophage and polymorphonuclear leukocyte (PMN) populations. The macrophage has an important role in genetically defined ntigen-driven immune activation and tumor-cell killing in addition to its phagocytic role. Defects in stem cell differentiation into PMN's or macrophages will predispose the host to bacterial infection and in the case of a macrophage defect will interrupt normal B- and T-lymphocyte activation.

As should be clear from the above discussion, toxicological alterations may affect the immune system in a very subtle or gross fashion depending upon the level of interference. If a single T- or B-cell subpopulation is affected, the loss of overall responsiveness and increase in disease susceptibility may be minimal. Such a loss of response may also be transient. However, an alteration in stem cell, thymus or bone marrow differentiative pathway may result in severe nonreversible impairment. It is important, therefore, to study a wide range of parameters in order accurately to assess any effect on the immune system as a whole.

Assessment of Immunologic Potential

Various immune assay systems measure different levels of cell involvement and complexity in response to a stimulus. As such, assays that measure responses that are a result of complex cell interaction mechanisms are more likely to reveal alterations in immune function. For this reason, one can order the immunologic parameters into a tier structure, based on sequential levels of cellular cooperation reflecting this degree of complexity. It is anticipated that studies of drug- or chemical-induced immune alterations would focus on the complex assay systems first. Once a drug-induced lesion is found, the cause can be dissected using assays that involve less complex systems. Such an approach will ultimately lead to the assays that will pinpoint the cellular basis of dysfunction. Table 1 shows one possible tier structure. This tier system is not meant to be exhaustive but to serve as an example of one possible experimental design. The components of Tier I measure reactions that depend upon complex multicellular interactions involving T and B lymphocytes and macrophages. Genetically controlled regulatory networks play an important role in these responses. It would be anticipated that immune alterations at one of several sites in the cell interaction network would result in a modified response. However, since these systems are complex, it may be possible to compensate for an immune lesion by using an alternative response pathway. Furthermore, since these systems only assess a limited area of the total immune system several should be employed to provide good coverage.

The assay systems in Tier II involve cell interaction mechanisms that are less complex than those in Tier I, often inducing only MΦ–T-cell or B-cell–MΦ–suppressor T-cell interactions. These assay

Table 1. Hierarchy of some immunologic assay systems.

Tier	Reaction
Tier I	Complex cell interactions
	In vivo spleen cell IgM and IgG responses to thymic-dependent antigens
	Popliteal lymph node proliferation following immunization
	Delayed cutaneous hypersensitivity
	Quantitation of circulating antibody levels following immunization with thymic-dependent and thymic-independent antigens
Tier II	Immunologic interactions of intermediate complexity
	Determination of basal immunoglobulin levels
	Mitogenic stimulation of T-lymphocytes with concanavalin A or phytohemagglutinin
	Stimulation of B-cell proliferation and antibody synthesis by *E. coli* lipopolysaccharide
	One-way mixed lymphocte reactivity
	Enumeration of thioglycollate-induced peritoneal exudate cells
Tier III	Direct cellular assays
	Determination of resident mouse peritoneal exudate cells
	Determination of phagocytic capacity of peritoneal exudate cells
	Cytostatic and cytotoxic capability of adherent normal and activated resident peritoneal exudate cells against malignant cells
	Reticuloendothelial system clearance

systems allow a more defined evaluation of a discrete portion of the immune response. As such they may allow a chemically induced effect detected in a Tier I assay to be localized to a particular cell type. Further definition of a toxic effect may be accomplished using direct cellular assays like those presented in Tier III.

Assays shown in Tier III represent some of the assays available to determine the function of a distinct cell population. Once it is suspected that a discrete cell population is affected by a chemical, assays of this type are useful in supporting the initial findings. By ordering the experimental protocols in a tier structure, a drug-induced alteration in immune responsiveness is likely to be rapidly detected and efficiently defined.

Conclusion

The immune response offers a well-defined system in which to study the toxic effects of suspected drugs or chemicals. Through many years of immunologic research our understanding of the complexity of the cellular interactions involved in any immune response allows us to ask specific toxicological questions. The following considerations are important in any immunotoxicological study. The immune system represents a complex multicellular, multiorgan network that may be affected at several sites. Alterations in one arm of the response (humoral or cellular) may not affect responses governed by the other limb. The immune system shows a great deal of resiliency and harmful effects may be transient. Some components of the immune system are long-lived (T-cells), whereas others are not (B-cells). Alterations in short-lived, replaceable systems may have little long-term consequence to the host. Immune response capability and the influence of various organs (thymus) changes with ages. Therefore deleterious effects may be a function of age. Since many immune responses are genetically controlled, alterations in responsiveness to one challenge in a given animal model may not hold true in second animal model. Genetic differences may also be seen between sexes.

In view of the above considerations it is important in any toxicological study of immune capacity to consider the immune system as a whole and design experimental protocols that consider the dynamic complexity of the system.

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Environmental Health Perspectives
Vol. 43, pp. 9-19, 1982

Diseases Associated with Immunosuppression

by Eugene R. Heise*

Impairment of any of the major components of the immune system (T-cells, B-cells, phagocytes, complement) may result in clinical immunodeficiency. Immune defects can arise from intrinsic or heritable defects of lymphoid elements, failure of normal cellular differentiation, viral infection or other acquired causes. Clinical impairment of immunity is expressed as a marked susceptibility to opportunistic and pathogenic organisms which are difficult to control and by an increased risk of malignancy, allergy and autoimmune disease. Certain immunodeficiency disorders are associated with aberrant immune regulation. The major types of immune deficiency are characterized by unique patterns of infections depending on the level at which the defect occurs and the pathogenic mechanisms of the parasite. The basic defects of representative primary and secondary immunodeficiencies are discussed in relation to observed immunologic consequences.

Overview

Normal host defenses require the participation of four major immune systems: T-lymphocytes, B-lymphocytes, phagocytes and complement. Diseases associated with immunologic deficiency comprise a large group of disorders which are heterogeneous in their clinical expression and in their pathogenesis. It is possible to categorize immunodeficiency disorders in several ways (*1*). However, no completely satisfactory classification has thus far emerged. One approach is to distinguish between primary and secondary immunodeficiency (Table 1). Primary immunodeficiencies originate from a defect within one or more of the four immune systems. This group generally is subdivided into disorders of the T-cell system, the B-cell system or both B- and T-cells. Primary immunodeficiencies typically are hereditary and are manifest early in life as repeated infections caused by microorganisms which produce infections in normal children that are not serious. Secondary immunodeficiency may be defined as a disorder affecting host defenses in which the initiating factor arises outside of the immune system or is not restricted to immune elements. Secondary immune impairment occurs more frequently than primary immunodeficiency and commonly occurs in an older age group. Adenosine deaminase (ADA) and nucleoside phosphorylase (NP) deficiency can be considered in this category since the biochemical defect is not limited to lymphocytes. Excessive loss of lymphocytes or immunoglobulin from the gastrointestinal and genitourinary tracts or the lymphatic system due to congenital malformations, to surgical accidents, to disease or to other cause can also depress immunity. Another common cause of immunosuppression is infectious disease, especially viral disease, which impairs the function of lymphocytes or the development of lymphoid organs. The widespread use of immunosuppressive agents and cytotoxic drugs in recipients of organ transplants, cancer patients and patients with autoallergic disorders can lead to states of severe immunologic deficiency. Immunologic recovery following irradiation may require as long as a year. In protein-calorie malnutrition, a marked lymphopenia develops and defects in lymphocyte function may occur.

Recent advances have greatly improved our understanding of immunodeficiency disorders. The advances include (1) the ability to isolate and identify distinct subpopulations of lymphoid cells, (2) the demonstration of functionally separate subpopulations of lymphocytes with unique surface antigens (2-5) and (3) the development of functional assays for the different lymphocyte subpopulations. These advances now permit immunologic defects to be localized to a particular developmental or regulatory pathway. Similar advances in leukocyte biology have identified and localized many defects in neutrophils, macrophages and the complement system. It should be emphasized that the various immune systems are closely integrated such that a defect in one system may have effects in other systems. Thus, deficient T helper cells may result in poor antibody responses. Improved understanding of the precise site of the defects in immune mechanisms could lead to a new classification of immunodeficiency based on mechanisms (Table 1). Already it is possible to distinguish several mechanisms of defects in specific and nonspecific immunity. For example, the defect can be intrinsic if an abnormality exists in hemopoeisis, lymphopoeisis or enzyme production, or there may be a failure in the differentiation pathway leading to effector T-cells or to antibody-secreting B-cells, resulting from abnormalities of the microenvironments which provide the short-range signals for T- and B-cell differentiation. Another likely possibility is that of disturbances of immune regulation involving T-cells, B-cells and macrophages. A second group of functional disorders are those that involve the nonspecific defense mechanisms. This latter group would include intrinsic defects in phagocytes affecting motility, phagocytosis or microbicidal mechanisms and defects in the system of complement proteins.

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Table 1. Categories of impaired immune function.

Category
Primary immunodeficiency
Defects of T-lymphocytes
Defects of B-lymphocytes
Combined T- and B-cell deficiency
Secondary immunodeficiency
Multisystem biochemical defects
Loss of lymphocytes or immunoglobulin
Immunosuppressive infections
Chemical or physical immunosuppression
Nutritional deficiency
Neoplasia
Defects of specific immune mechanisms
Intrinsic defects of lymphoid cells
Impaired differentiation of lymphoid cells
Disorders of immune regulation
Defects of nonspecific defense mechanisms
Defects in phagocytosis or microbicidal killing
Defects of the complement system

The principal manifestations of impaired host defenses are recurrent and severe infections caused by opportunistic microorganisms. These infections are difficult to treat with appropriate antibiotics. The types of infections provide important clues to the particular system(s) deficient in function (Table 2). For example, recurrent infections with encapsulated pyogenic bacteria could indicate a defect in either the B-cell system (immunoglobulin deficiency), the phagocyte system or the complement system. In contrast, patients with defective T-cell-mediated systems are especially susceptible to infections by intracellular parasites including fungi, bacteria and viruses, which present as pneumonia or as chronic infections of the skin, mucous membranes or other tissues. Complications of combined B- and T-cell deficiency generally are more serious than those caused by B-cell deficiency.

Table 2. Some complications of immunodeficiency.

Defective system	Etiology	Pattern of infectious complications
T-cells	SCID, thymic hypoplasia, treated malignancy, immunosuppression	Severe viral infections (chicken pox, measles, cytomegalovirus, herpesvirus) Pneumonia caused by *Pneumocystis carinii* Opportunistic fungi (Candida, Aspergillus, Nocardia) Intrauterine or post-transfusion graft-versus host disease
B-cells	IgG, IgM, or IgA deficiency	Severe infections with pyogenic bacteria, hepatitis virus, ECHO virus, live poliovirus vaccine and protozoa (Giardia)
Polymorphonuclear leukocytes	Defective killing	Severe and/or recurrent pyogenic skin infections Superficial fungal infections Chronic osteomyletis (Klebsiella and Serratia sp.)
Complement		Severe and/or recurrent pyogenic infections

Immunodeficiency Associated with Intrinsic Defects in Lymphoid Cells

The most profound of the immunodeficiency seases are included in a heterogenous group ferred to as severe combined immunodeficiency CID), which is characterized by disorders in th the T-cell and B-cell systems (Table 3). The ost severe of these is reticular dysgenesis arising om a defect at the level of hematopoietic stem lls which results in the virtual absence of T-cells, -cells and neutrophils. In the Swiss type of CID, lymphoid cells fail to develop, although ematopoietic stem cells are normal. T- and B-cells re greatly diminished, and neither responds to ntigenic stimulation. Another type is SCID associated with a deficiency of adenosine deaminase (ADA). This enzyme deficiency prevents the conversion of adenosine to inosine and deoxyadenosine to deoxyinosine, resulting in an accumulation of deoxy ATP, which is toxic, especially to lymphocytes. Some patients with SCID have normal numbers of B-cells. However, T-cell-mediated immunity is almost always deficient. Some cases of ADA deficiency have been reconstituted with red blood cell transfusions (*7*). The ADA in the red cells reduced the level of deoxy ATP and restored lymphocyte function. ADA-deficient SCID patients also have been successfully treated by bone marrow transplantation. Recently, Gelfand et al. (*8*) described a form of SCID with normal numbers of B- and T-lymphocytes. The lymphocytes from these patients failed to proliferate when stimulated *in vitro*. Investigation revealed that the lymphocytes were defective in lateral mobility of membrane receptors (frozen membrane) possibly caused by a defect in the assembly of cytoplasmic microtubules.

Another enzyme deficiency involves the enzyme rine nucleoside phosphorylase (PNP) which basily is a condition with impaired T-cell function t with normal B-cell function. The enzyme PNP alyzes the conversion of inosine to xanthine and of guanosine to guanine in the purine catabolic pathway. The immunologic abnormalities in PNP deficiency consist of diminished delayed hypersensitivity responses, reduced numbers of circulating T-cells and reduced *in vitro* proliferative responses to mitogens, antigens and allogeneic cells. Serum immunoglobulin levels and antibody responses are normal. Evidence has been presented which suggests that deoxyguanosine triphosphate is a toxic metabolite responsible for the immunodeficiency (*9*).

Immunodeficiencies Involving Impaired Differentiation of Lymphoid Cells

Disorders thought to involve impaired maturation of T- and B-cells are listed in Table 4. Thymic hypoplasia (DiGeorge syndrome) is believed to result from an undefined intrauterine insult affecting the third and fourth pharyngeal pouches. Consequently, the microenvironment necessary for T-cell development fails to develop. Patients with the DiGeorge syndrome have increased susceptibility to sinusitis, recurrent pneumonia and diarrhea. The infections are often caused by *Candida albicans* and protozoa. The absolute lymphocyte count is usually normal, with a reduced percentage of T-cells and an increased percentage of B-cells. T-cell-mediated functions are decreased markedly, whereas B-cell functions are remarkedly normal, considering that T-cells are required for initiation of immune responses to most antigens. Fetal thymus transplants have resulted in rapid restoration of T-cell function even when the thymus transplant was enclosed in a millipore filter, which suggests that a lack of thymic hormones may be responsible for this disease.

X-linked agammaglobulinemia is manifest by early onset of recurrent infections with encapsulated pathogenic bacteria (i.e., staphylococci, pneumococci, streptococci and hemophilus) and with several viruses, specifically, hepatitis virus and enterovirus (ECHO virus and poliovirus). Patients

Table 3. Immunodeficiency diseases involving intrinsic defects in lymphoid cells.[a]

nical diagnosis	Presumed level of basic defect	Immunologic defect	
		Cellular abnormalities	Functional deficiencies
CID, reticular dysgenesis	Hematopoeitic stem cell	T-, B-, and phagocytes	CMI, antibody, phagocytosis
ID, Swiss type	Lymphocytic stem cell	T-, B-cells	CMI, antibody
ID with ADA deficiency	Adenosine deaminase deficiency	T ± B cells	CMI, antibody
rine nucleoside phosphorylase deficiency	Absent or abnormal enzyme	T-cells	CMI

After Waldmann et al. (*6*).

Table 4. Immunodeficiency diseases involving impaired differentiation of lymphoid cells.[a]

Diagnosis	Presumed level of basic defect	Immunologic defect	
		Cellular abnormalities	Functional deficiencies
Thymic hypoplasia	Failure of thymic development	T	CMI± antibody
X-linked agammaglobulinemia	Failure of pre-B maturation ± suppressor T-cells	B	Antibody
Transcobalamine II deficiency	Failure of B-cell differentiation, vitamin B_{12} deficiency	Plasma cells	Antibody and phagocytosis
Common variable immunodeficiency (one type)	Failure of pre-B to B-cell maturation	B	Antibody
Ataxia telangiectasia	Thymic failure and failure of terminal maturation of B_α, B_ϵ cells	T, IgA, IgE plasma cells	CMI ± antibody
Transient hypogammaglobulinemia (infancy)	Impaired terminal differentiation of B-cells	Plasma cells	Antibody
Selective IgA deficiency	Failure of terminal differentiation of B_α	IgA plasma cells	IgA antibody

[a] After Waldmann et al. (*6*).

with this disorder exhibit low concentrations of all immunoglobulin classes except IgE. These patients lack circulating B-cells and plasma cells in stimulated nodes. In contrast, the thymus is normal, and normal thymic-dependent areas are present in peripheral tissues. Evidence has been obtained which indicates that the defect occurs at the level of the transition from pre-B-cells to B-cells. Dosch et al. (*10*) demonstrated that a patient with X-linked disease possessed precursor B-cells which could develop surface IgM after a period of *in vitro* culture and that the transition from pre-B-cells to antibody-producing cells could occur *in vitro*.

Deficiency in transcobalamin II, a protein important in transport of vitamin B_{12} in the blood, is associated with normal numbers of circulating B-cells and T-cells (*11*). However, plasma cells are absent and virtually no immunoglobulins or specific antibodies are produced. Following vitamin B_{12} therapy the patient is able to make antibodies, suggesting that vitamin B_{12} is necessary for the transformation of B-cells into immunoglobulin-producing plasma cells.

Common variable immunodeficiency comprises a heterogeneous group of disorders associated with high incidence of infections caused by encapsulated extracellular organisms and reduced immunoglobulins. One subgroup of common variable immunodeficiency apparently is caused by an intrinsic defect in B-cell differentiation. This inference is based on the demonstration that *in vitro* cultures of whole or separated cell populations stimulated with polyclonal B-cell mitogens did not synthesize or secrete immunoglobulin molecules (*12*).

Ataxia-telangiectasia is associated with recurrent sinopulmonary infections and an increased incidence of malignancy especially lymphoreticular tumors. Abnormalities of both T-cell and B-cell systems are present in most patients. The most prominent abnormality in the B-cell system is a deficiency of IgA and/or IgE. B-cells bearing surface IgA are present, which indicates that the defect probably is at the level of terminal differentiation rather than an inability to synthesize the alpha chain (H chain) of IgA.

Selective IgA deficiency is manifested by respiratory and gastrointestinal disease as can be expected because IgA plays an important role in humoral immunity of mucosal surfaces. Moreover, IgA deficiency is associated with an increased risk of allergy, autoimmune disease and carcinoma. Antibodies to IgA are relatively frequent in individuals lacking this immunoglobulin. In the majority of patients an intrinsic defect in the B-cell system leads to failure of IgA bearing cells to develop into IgA synthesizing and secreting plasma cells (*13*). An excess of IgA suppressor cells does not seem to play a role in IgA deficiency.

Disorders Involving Abnormalities of Immunoregulatory Cells

Aberrant immunoregulation is postulated for several autoimmune diseases (*14*) and immunodeficiency. Data supporting this hypothesis have been obtained by various *in vitro* methods including: determination of T_M (IgM Fc receptor) and T_G (IgG Fc receptor) bearing T-cells, mitogen-driven B-cell immunoglobulin synthesis and use of monoclonal antibodies to T-cell differentiation antigens. Using monoclonal antibodies, Reinherz and colleagues (*15*, *16*) demonstrated disturbances in the normal immune homeostatic mechanisms in several immunodeficiency diseases, autoimmune disorders and other conditions (Table 5). Four different patterns of T-cell disturbances have been distinguished. The first type consists of a deficiency of helper T-cells, which is observed in one type of common variable immunodeficiency. A second group of conditions includes acute graft versus host disease (GVH), scleroderma, Sjögren's disease, hemolytic anemia and sarcoidosis. This group is reported to exhibit excessive numbers of activated helper T-cells and reduced numbers of suppressor T-cells. The third pattern is that of reduced numbers of suppressor T-cells with adequate numbers of helper T-cells. This pattern is consistent with results obtained by others who have enumerated the T_M (helper) and T_G suppressor subsets or have used mitogen-driven antibody secretion assays for functional studies. A fourth pattern is that of quantitatively increased numbers of activated suppressor T-cells. In this subset of common variable immunodeficiency, the suppressor T-cell abnormality probably plays a primary role. This is suggested by experiments in which purified B-cells and monocytes from which T-cells had been removed were able to synthesize immunoglobulins *in vitro*. Moreover, unseparated lymphocytes from these patients synthesized IgM normally when they were cultured in the presence of hydrocortisone succinate, a drug which inhibits activation of suppressor cells (*13*).

Immunodeficiency Associated with Lymphoreticular Malignancies

Neoplasms of B- and T-cells frequently are associated with disorders of cellular or humoral immune mechanisms. These immunodeficiencies are of particular interest in relation to mechanisms of immune regulation. Several examples of B-cell and T-cell malignancies will be considered.

Chronic lymphoblastic leukemia (CLL) is a neoplasm characterized by the accumulation of excessive numbers of small lymphocytes in the blood, bone marrow or other tissues. In more than 95% of cases, the cells express surface immunoglobulin that is restricted as to heavy-chain type, light-chain type and antibody specificity. These findings indicate that the CLL cells are clonally derived. CLL frequently causes a secondary immunodeficiency which is characterized by decreased humoral responses and immunoglobulin levels together with some diminution of cell-mediated immunity (*17*). Decreased synthesis is the major factor in the reduced serum immunoglobulin levels (*18*). The cause of the decreased immunoglobulin production has not been defined, but does not appear to be caused by excessive suppressor T-cell activity (*19*).

Multiple myeloma is a disease characterized by monoclonal proliferation of plasma cells in the bone marrow and lytic bone lesions. Usually, serum or urine monoclonal myeloma paraproteins are present. The paraproteins represent a homogeneous population of immunoglobulins or Ig subunits, especially light (L) chains (kappa or lambda). In some cases myeloma proteins are found to have specific antigen binding activity, often directed against phospholipid moieties common to cell membranes.

In certain animal models, the proliferation of monoclonal plasma cells has been found to be regulated by T-cells and macrophages, similar to the amplication and suppression of normal B-cell differentiation. The function of residual polyclonal B-cells is often markedly impaired in patients with multiple myeloma leading to depression of humoral immunity and to reduced heterogeneity of Ig molecules in immunoelectrophoresis determinations. To a lesser extent, nonspecific host defenses and cell-mediated immunity also are impaired (*20, 21*). A summary of the immunologic abnormalities is shown in Table 6.

Table 5. Disorders of human T-cell subsets defined by monoclonal antibodies.

Disease	Presumed abnormality	Phenotype[a]		
		$T4^+$	$T5^+$	I_A^+
Subset of patients with common variable immunodeficiency	Deficient T_H cells	↓	*n*	*n*
Acute GVH, scleroderma, Sjogrens, sarcoid, hemolytic anemia	Excessive T_H cells (act.) with deficient T_S cells	↑	↓	↑
Systemic lupus erythematosis	Deficient T_S cells	*n*	↓	*n*
Subset of patients with common variable ID; infectious mononucleosis, cytomegalovirus, chronic GVH	Excessive T_S cells (act.)	*n*	↑	↑

[a] *n* = normal numbers of a T-cell subset; I_a^+ = nonpolymorphic determinant of I-region associated-like protein; T_4 = T_H cell antigen defined by a monoclonal antibody; T_5 = T_S cell antigen defined by a monoclonal antibody.

Table 6. Immunologic features of multiple myeloma.

- Humoral immunity
  - Serum paraproteins (80%)
  - Urinary paraproteins (50%)
  - Moderately reduced levels of nonmyeloma immunoglobulins
  - Reduced or absent natural antibodies
  - Impaired antibody formation
  - Decreased synthesis and increased catabolism of immunoglobulins
  - Reduced numbers of circulating B-cells
  - Suppression of mitogen-induced immunoglobulin synthesis
- Nonspecific host defenses
  - Reduced levels of serum complement ($C1_q$, C2, C4)
  - Decreased granulocyte migration and adhesiveness
  - Decreased granulocyte lysozyme concentration
- Cell-mediated immunity
  - Decreased ability to develop primary DH responses
  - Inconsistent depression of delayed hypersensitivity to common antigens
  - Infectious complications

Recurrent bacterial infection is a common complication of multiple myeloma. Bacterial infection is the presenting feature in about 25% of myeloma patients (*22*) and may be the immediate cause of death (*23*). The major type of infections are pneumonia caused by pneumococci or staphylococci and urinary tract infections caused by gram-negative organisms (*E. coli*, *Pseudomonas*, *Proteus* and *Kelbsiella*) (*24*). The major immunologic defect observed in myeloma patients is in the humoral system. Broder and Waldmann (*25*) recently concluded that at least one mechanism for the humoral deficiency observed in myeloma patients is a block in polyclonal B lymphocyte maturation and immunoglobulin synthesis. However, these patients also have defects in antigen nonspecific host defenses that include dysfunction of granulocytes and of the complement system. These authors also conclude that if a clinically significant abnormality of cellular immune function exists, it is smaller in magnitude compared to deficiency of humoral immunity. In one study, untreated myeloma patients were found to have circulating suppressor cells that inhibited mitogen-induced immunoglobulin synthesis (*26*). The suppressor cells were Fc receptor-positive and nonphagocytic.

Hodgkin's disease is a malignant lymphoma which is associated with increased incidence of infection (*27*) and defects in T-cell-mediated immunity (*28*). The abnormalities of cell-mediated immunity and lymphocyte function are seen in a proportion of untreated cases with early disease (stage I and II). Moreover, the degree of depression is related to the clinical stage of disease, i.e., deficiency is more marked in advanced stages of disease (stage III and IV), independent of treatment (radiotherapy or chemotherapy) which is itself immunosuppressive. Pretreatment absolute lymphocyte counts are significantly correlated with clinical stage, skin test reactivity and life expectancy in Hodgkin's disease (*29*, *30*). Reduced skin test reactivity to common microbial antigens or to primary sensitization with dinitrochlorobenzene (DNCB) is commonly observed (*31-33*). Decreased delayed hypersensitivity is not specific for any single antigen (anergy). In some cases, lymphocyte function as assessed by lymphocyte proliferation *in vitro*, is reduced at only low PHA concentrations (*34*). In more severe disease, decreased proliferative responses to all concentrations of PHA are observed (*35*). Significant reduction in the percentages of E-rosette-forming cells (T-cells) is an inconsistent finding (*36*, *37*) which may be related to the occasional finding that patient plasma or serum can inhibit E-rosette formation (*38*) and lymphocyte transformation (*39*). The inhibitors present in plasma from active Hodgkin's patients may be due to the presence of T-cell antibodies (lymphocytotoxins) (*40*) or nonimmunoglobulins, especially lipoproteins.

Several investigators have suggested that excessive suppressor cell activity may be responsible for the immune deficiency in Hodgkin's disease. For example, Engleman et al. (*41*) reported that 41 of 70 (58%) of Hodgkin's disease patients had a disproportionate number of circulating suppressor cells capable of inhibiting mixed lymphocyte reactions. Goodwin et al. (*42*) obtained data indicating that the suppressors are glass-adherent, prostaglandin E_2-producing mononuclear cells, which suggests that macrophages are responsible for the suppression. Interestingly, a defect in the ability of peripheral monocytes from patients to ingest and kill Candida organisms by a myeloperoxidase independent system was reported recently (*43*). The data strongly suggest that abnormal functioning macrophages are important in the pathogenesis of immunodeficiency in Hodgkin's disease.

Several neoplasms of immunoregulatory T-cells have been investigated in recent years. Sezary' syndrome is a form of T-cell lymphoma wit circulating malignant cells which have a propensit for infiltrating the epidermis. About one half o Sezary syndrome patients have a homogeneou population of neoplastic but functional helper T-cell (*44*). Another interesting malignant disease o regulatory cells is a T-cell leukemia of adult onse which has been reported in Japan, near the site o an atomic bomb explosion on the island of Kyushu. In these cases there was no mediastinal mass. Th leukemia cells were identified as T-cells on th basis of E-rosette formation and by cytotoxicit using a heterologous T cell antiserum. Japanese adult T-cell leukemia differs from Sezary syndrome in that typical Sezary cells are absent, cellular infiltration of the skin occurs in the dermis rather than the epidermis and infiltration of the bone marrow occurs.

Immunodeficiency Associated with Infectious Disease

Specific immunity to several viral diseases, such as herpes, measles, rubella and vaccinia depends on T-cell-mediated immunity. Primary or secondary immunodeficiency of the T-cell system is associated with recurrent and often fatal viral disease, as previously noted. Conversely, certain viruses themselves are immunosuppressive when they infect immunocompetent cells. The best examples of immunosuppression secondary to viral disease are the paramyxovirus infections (measles, mumps and influenza). In these viral infections, delayed hypersensitivity is depressed within the first few days and persists for several weeks. The exact mechanism responsible for anergy following measles infection remains uncertain. However, at least three mechanisms can be considered. First, measles virus infection produces a lymphopenia, leaving fewer effector cells available for eliciting delayed hypersensitivity reactions. Second, measles virus can infect stimulated and proliferating lymphocytes and thus may directly interfere with the function of lymphocytes which are engaged in specific immune responses. Third, measles virus may compete with antigen for receptors on the lymphocyte surface.

Depressed delayed hypersensitivity is also associated with certain bacterial and fungal infections, especially disseminated and granulomatous infections with intracellular organisms. Classic examples are miliary tuberculosis and active pulmonary tuberculosis, coccidiomycosis, histoplasmosis, blastomycosis and lepromatous leprosy (*46*). Anergy has been significantly associated with both lymphocytopenia and leukocytosis. However, functional abnormalities of lymphocytes or macrophages probably are responsible for anergy in these diseases. Several laboratories have studied the regulation of delayed hypersensitivity in experimental animal models (*47-49*). Transient suppression of delayed hypersensitivity can be accomplished by administration of specific soluble antigen to animals with established skin test responsiveness. Antigen-induced suppression of delayed hypersensitivity exhibits several features that resemble that seen in anergy. Animals sensitized to multiple antigens and challenged with mg quantities of a single specific antigen exhibit a profound, but transient, period of complete anergy which may persist for several days or weeks. This period of nonspecific unresponsiveness is followed by a second phase of hyporesponsiveness which is specific for the challenge antigen (*49*). Dwyer et al. (*48*) obtained evidence which strongly suggests that lymphocytes of a desensitized animal are competent but are actively suppressed *in vivo*. These investigators suggested that the products of stimulated suppressor T-cells exert a local effect on bystander lymphocytes (*50*). Presumably both specific and nonspecific suppressor factors are active at different times in order to explain both phases of anergy.

More recently, the relationship between granuloma formation and delayed hypersensitivity was studied in our laboratory (*51*). The results of this study indicated that dermal delayed hypersensitivity and allergic granuloma formation in the skin are both suppressed in sensitized animals following systemic challenge with specific antigen. In this model, pulmonary granulomas were unaffected. This data indicated to us that delayed hypersensitivity and allergic granulomatous responses which depend on circulating T-cells are controlled by the same regulatory system.

Immunodeficiency Associated with Autoimmune Disease

Immunodeficiency can occur in patients with B-cell hyperreactivity as in systemic lupus erythematosis (SLE). An increased risk of infection prior to therapy is likely, although firm evidence is lacking. Pyelonephritis, pneumonia and septicemia are relatively common. Organisms causing these infections typically are pyogenic bacteria, especially Staphylococci. Although the basis of this predilection of infections is uncertain, possible explanations could include low serum complement levels, abnormal leukocyte function and abnormalities in regulatory T-cells. Individuals with IgA deficiency seem to be predisposed to develop autoantibodies and autoimmune disease. This association may be the result of an inability of the host to prevent the entrance of antigens from mucous surfaces that are sufficiently similar to autologous antigens to induce an immune response to autologous tissue antigens or both.

Disorders Involving Excessive Loss of Immunoglobulins and Lymphocytes

Hypoimmunoglobulinemia may result from loss of protein into the urinary and gastrointestinal tract (Table 7). Loss of protein into the urine occurs when there is a defect in the renal glomerular membrane or in renal tubules. In nephrotic syndrome there is selective loss of the 7S immunoglobulins (IgG, IgA, etc.) with little or no loss of 19S IgM.

Protein can also be lost into the gastrointestinal tract; however, this rarely leads to clinically evident immunodeficiency. Intestinal lymphoangiectasia is an exception because in this disease, loss of immunoglobulins is accompanied by loss of lymphocytes secondary to abnormalities of lymphatic channel development or other disease processes. Patients with this disorder have abnormalities in both cellular and humoral immunity. Serum concentrations of IgG, IgM and IgA are reduced although the rate of synthesis is normal. The defect in cell-mediated immunity is more profound than that in humoral immunity. These patients become markedly lymphopenic and are depleted in long-lived lymphocytes, which is analogous to thoracic duct drainage, a well-known and effective immunosuppressive procedure.

Disorders of Neutrophilic Phagocytes

Neutrophils are short-lived cells which are specialized for ingestion and destruction of certain classes of microorganisms. Failure of chemotaxis (directed locomotion), phagocytosis or intracellular killing results in increased susceptibility to opportunistic infections with bacteria; however, patients do not generally experience difficulties with viruses or protozoa.

Disorders of neutrophil function may be divided into intrinsic and extrinsic defects (Table 8). In chronic granulomatous disease (CGD), granulocytes and monocytes are deficient in NADH or NADPH oxidase or NADPH reductase. The defect in killing appears to be at the level of post-phagocytic oxygen metabolism involved with the generation of hydrogen peroxide (H_2O_2) or O_2^-. Organisms causing infection in CGD are almost always catalase producers (e.g., *Staphylococcus aureus*, *Serratia marscens*, *Aspergillus*). Catalase-producing organisms rapidly destroy hydrogen peroxide, whereas catalase-negative organisms, such as pneumococci do not destroy the available hydrogen peroxide of the phagocyte which the cell uses to effect microbial killing, most likely by interaction with myeloperoxidase and halide (*52*).

Chediak-Higashi syndrome is a rare multisystem disorder. The striking morphologic disorder consists of giant cytoplasmic inclusions derived from azurophilic granules (lysosomes) which are present in many cells, including granulocytes and monocytes. Both types of phagocytes are deficient in microbicidal and chemotactic activity. A primary defect in tubulin affecting microtubule polymerization and degranulation has been suggested as the basis of the disorder.

Extrinsic causes of neutrophil dysfunction are numerous and are often related to leucopenia or decreased opsonization, abnormal chemotaxis or other defects. When the total number of functioning neutrophils is reduced below a critical level (~ 1000/μl), infection is likely to ensue. Leukopenia is frequently drug-induced. Anti-inflammatory drugs (e.g., corticosteroids) characteristically induce abnormalities in neutrophil adherence and migration in addition to many other actions. Another mechanism is the premature destruction of neutrophils resulting from formation of neutrophil antibodies, as in autoimmune neutropenia, which may cause removal of neutrophils via antibody-dependent lymphocyte-mediated cytotoxicity (ADCC). In drug-induced immune neutrophenia, the cells may absorb drug-antibody immune complexes which may result in neutrophil destruction via an innocent bystander mechanism.

Abnormal phagocytosis can be secondary to a large number of other disorders. In diabetes, for example, chemotaxis and bacterial ingestion is decreased. Decreased phagocytic function is also

Table 7. Disorders involving excessive catabolism or excretion.

Diagnosis	Presumed level of basic defect	Immunologic defect
Familial hypercatabolic hypoproteinemia	Hypercatabolism of serum proteins	Decreased Ig levels
Myotonic dystrophy	Hypercatabolism of IgG	Decreased IgG levels
Nephrotic syndrome	Sieving property of glomerular membrane	Decreased IgG levels
Intestinal lymphangiectasia	Disorder of lymphatic channels with loss of lymph fluid and cells	Diminished CMI ± A

Table 8. Neutrophil disorders.

Intrinsic	Extrinsic
Enzymatic defects	Leukopenia
Defective O_2^- and H_2O_2 generation	Drugs and toxins
Chronic granulomatous disease	Adrenocorticosteroids
G6PD deficiency	Ethanol
Myeloperoxidase deficiency	Aminopyrine, phenylbutazone
Degranulation defects	Immune
Chediak-Higashi syndrome	Neutrophil antibodies
Locomotion and chemotasis defects	Radiation
Lazy leukocyte syndrome	Disease
	Aplastic anemia, acute leukemia
	Deficiency of opsonins
	Immunodeficiencies of Ig or complement
	Neoplastic disease
	Multiple myeloma
	Chronic lymphocytic leukemia

secondary to burns, alcoholic cirrhosis malnutrition and sickle cell disease.

Myeloperoxidase deficiency in leukocytes can be hereditary or secondary to refractory anemias or leukemias. In some cases, an increased susceptibility to Candida or staphylococci have been noted. Myeloperoxidase reduces hydrogen peroxide and in the presence of intracellular halide (iodine) leads to production of a lethal substance, as previously mentioned. The intracellular killing of these organisms *in vitro* is delayed compared to normal leukocytes.

Disorders of the Complement System

The physiologic role of complement is clearly illustrated by the diseases which occur in complement deficiency states or when complement is activated under inappropriate circumstances. Patients with deficiencies in early components of the classic pathway (i.e., Clr, Cls, C4, and C2) have increased susceptibility to systemic lupus erythematosis or other collagen vascular diseases. This finding may indicate the role of complement in efficient destruction of viruses or in the removal or inactivation of some types of immune complexes. Complement C3 and C3b deficiency is associated with recurrent infections with pyogenic bacteria. About half of patients with deficiencies of the terminal components, i.e., C5, C6, C7 and C8, have recurrent infections with Neisseria organisms (gonococci and meningococci), apparently because cytolysis with these organisms is complement-dependent.

In conclusion, clinically apparent immunodeficiency is associated with frequent and persistent infections. Recurrent infections with the same organism or with organisms of low pathogenicity together with an incomplete response to appropriate antibiotics are suggestive of impaired immunity. Other features of certain immunodeficiency disorders include an increased risk of malignancy, autoallergic disease and allergy. Specific hereditary immunodeficiencies manifest additional anatomic and constitutional features. Impaired T-cell immunity generally is more serious than deficient B-cell immunity. In secondary immunodeficiency, normal function generally is restored once the underlying disease or condition is corrected.

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Environmental Health Perspectives
Vol. 43, pp. 21-25, 1982

Chemotherapy-Induced Immunosuppression

by Lucy Rasmussen* and Ann Arvin*

Chemotherapeutic agents are used widely in clinical medicine for the treatment of conditions where diminution of the host immune response is a goal. The clinical use of immunosuppression is indicated for immunologically mediated disease, lymphoproliferative diseases, and prevention of graft rejection. Five categories of agents are useful for these purposes; they are ionizing irradiation, corticosteroids, biological alkylating agents, antilymphocyte sera and antimetabolites. While the specific molecular action of many of these drugs is known, how they affect cellular events in immune responses is less clear. One of the unfortunate sequelae of chemotherapy induced immunosuppression is an increased susceptibility of the host to opportunistic pathogens or malignancies.

Specific methods are described for monitoring the various parameters of both humoral and cellular immunity.

Studies of immunologic function in lymphoma patients and cardiac transplant patients treated with immunosuppressive drugs have shown specific defects in cell mediated immunity to herpes viruses which may relate to their increased susceptibility to infection by these agents.

Immunosuppressive Agents Used in Clinical Medicine

The use of immunosuppressive drugs in clinical medicine is necessary to diminish the immune response to a point where responses to undesirable foreign antigens are eliminated. This state of chemically induced immunologic unresponsiveness may have undesirable features, for example placing the host at risk of infection by a wide variety of opportunistic agents. This review will focus on three major areas: first, a broad review of immunosuppressive agents available in clinical medicine and clinical situations in which they are useful. Second, specific laboratory methods for assessing immunity will be emphasized. Last, model systems for studying the effects of immunosuppression will be analyzed.

A battery of immunosuppressive agents is available for use in clinical medicine. These are detailed in Table 1 along with examples of each category.

Table 1. Immunosuppressive agents available in clinical medicine.

Agent	Example
Ionizing irradiation	Whole body irradiation Extracorporeal irradiation
Corticosteroids	Prednisone
Biological alkylating agents	Nitrogen mustards Cyclophosphamide Chlorambucil, melphalan
Lymphocyte depletion by thoracic duct drainage or antilympocyte serum	Antilymphocyte sera
Antimetabolites	
Antibiotics	Azaserine Mitomycin C Actinomycins Chloramphenicol
Purine, pyrimidine antagonists	6-MP, azathioprine Cytosine arabinoside
Folate antagonists	Methotrexate
Alkaloids	Colchicine

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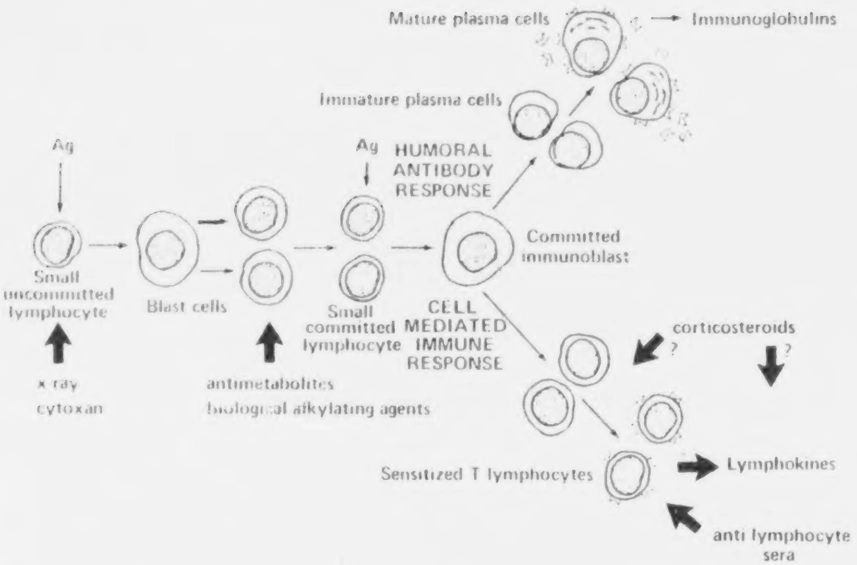
Mature plasma cells
Immunoglobulins
Immature plasma cells
Ag
Ag
HUMORAL
ANTIBODY
RESPONSE
Committed
immunoblast
Small
uncommitted
lymphocyte
Blast cells
Small
committed
lymphocyte
CELL
MEDIATED
IMMUNE
RESPONSE
corticosteroids
?
?
x ray
cytoxan
antimetabolites
biological alkylating agents
Sensitized T lymphocytes
Lymphokines
anti lymphocyte
sera

FIGURE 1. Schematic representation of stages in the immune response.

In general, these forms of immunosuppression are nonspecific and lead to diminution of response to a wide variety of agents. While methods of induction of unresponsiveness to specific antigens are available, they generally utilize either specific antibody or antigen. This area has been treated in detail in excellent reviews edited by Talmage and associates (*1*) and will not be considered here.

The modes of action of most immunosuppressive agents at the cellular level are complex and far from being clear at this point. Schematically, the events in an immune response may be divided into "inductive" and "productive" phases. The "inductive" phase involves the interaction of antigen with small lymphocytes. The "productive" stage involves steps resulting in the proliferation of stimulated cells with the eventual elaboration of products, for example, antibody for humoral immunity or sensitized T-lymphocyte and lymphokines for cell-mediated immunity. Most lines of evidence suggest that many immunosuppressive agents act mainly by impairing the reproductive capacity of immunologically competent or active cells. This may occur at multiple points in the cell cycle. Figure 1 schematically represents stages in the immune response culminating in both humoral and cellular immune responses. Irradiation at sublethal doses of 300 to 500 rads has variable effects on immune responses. Its primary site of action is probably at the level of destruction of the small lymphocyte population which is the precursor to events in immune proliferation. Cytotoxic drugs, for example antimetabolites and biological alkylating agents, act by interfering primarily with rapidly dividing cells. Alkylating agents will cross-link DNA strands. One exception is cytoxan, which may act by interfering with antibody at the stage of induction. Antilymphocyte sera are obtained from animals immunized with human lymphocytes and their main action appears to be on cell mediated immunity rather than antibody production. Corticosteroids are used primarily as anti-inflammatory agents, their main effect being stabilization of liposomal membranes within phagocytic cells. However, lympholytic activities as well as effects on T-cell–macrophage interaction have been ascribed to this group of drugs.

In general, nonspecific immunosuppressants are more effective at blocking the primary as compared to secondary or "anamnestic" immune response.

The use in clinical medicine of chemotherapeutic agents with immunosuppressive activity is directed at alleviating immunologically mediated diseases, lymphoproliferative diseases and prevention of graft rejections following organ transplantation. Some examples of agents useful in these respects are shown in Table 2. However, prolonged immunosuppression is dangerous: not only are the agents highly toxic for various cells but their use is associated with increased susceptibility to various infections (Table 3) and to secondary malignancies. Particularly disturbing is the increased frequency of cancer in kidney allograft recipients. Suppres-

Table 2. Clinical uses of immunosuppression.

Condition	Example	Agent
Immunologically mediated disease		
Exogenous antigens	Allergy	steroids
Homologous antigens	Rh isoimmunization	Anti Rh$_o$ globulin
Autologous antigens	Autoimmune disease	Steroids Antimetabolites
Lymphoproliferative diseases	Multiple myeloma Hodgkin's disease Leukemia	Steroids X-ray Antimetabolites
Transplantation	Cardiac and renal transplants	Antilymphocyte serum Steroids Antimetabolites

Table 3. Infections associated with chemotherapy-induced immune suppression.

- Viral
  - Herpesvirus infections, e.g., zoster, cytomegalovirus, Epstein-Barr
  - Viral vaccines, e.g., vaccinia
- Bacterial
  - Gram-negative organisms
  - *Staphylococcus aureus*
- Fungal/yeast
  - Aspergillus
  - Candida
- Malignancies of unknown etiology
  - Tumors in homograft recipients on immunosuppression
  - Second and third malignancies in leukemia or lymphoma patients on cytotoxic therapy, particularly with biological alkylating agents.
  - Carcinomas in congenital immunodeficiencies

Table 4. Assessment of humoral immune functions.

- Quantitation of serum immunoglobulins
  - Radial immunodiffusion
  - Serum electrophoresis
  - Immunoelectrophoresis
- Quantitation of specific antibody
  - Agglutination
  - CF
  - RIA
  - ELISA
- Specific antibody responses after immunization
- B-cell quantitation by immunofluorescence and transformation response to mitogens PWM

Table 5. Assessment of cellular immune function.

- Neutrophils
  - Quantitation
  - Phagocytosis: Nitroblue tetrazolium reduction
- Skin test for delayed hypersensitivity to specific antigens, e.g., TB
- T-lymphocytes
  - Quantitation: rosettes
  - Activation:
    - Transformation or DNA synthesis after mitogen, e.g., PHA or specific antigen stimulation
    - Lymphokines, e.g., migration inhibition factor, interferon
    - HLA restricted cytotoxicity

sion of cell-mediated rather than humoral immunity is thought to be primarily responsible, because the most effective antitumor responses are believed to be cell-mediated.

Specific Methods for Assessment of Immunity

Because of the known immunosuppressive action of many drugs used in clinical medicine, it is becoming of increasing importance to monitor immunologic parameters during therapy. Because immune function can be conveniently compartmentalized as either "humoral" or "cellular," it is operationally possible to assess functions in these respects. However, it must be emphasized that because of the interdependent nature of all immunologic responses, failure to detect reactivity at one level does not necessarily represent a primary site of action of a particular drug or drugs. Tables 4 and 5 list some specific methods that are useful in evaluating both humoral and cellular immunity. More detailed descriptions of these currently used methodologies are given elsewhere (*2*).

Model Systems for Studying Effects of Immunosuppressive Drugs in Patient Populations

At Stanford University Medical Center, there are two large populations of immunosuppressed patients. Patients are treated for Hodgkin's disease, as well as other lymphoid tumors, by irradiation and chemotherapy. Additionally, cardiac transplants have been performed at Stanford, and to reduce risk of graft rejection immunosuppression must be used in this group. Because of the frequent occurrence of infectious diseases in these two patient populations, we have instituted a program of prospective immunologic monitoring in order to attempt to identify those functions which may be useful prognostic indications of serious disease.

Herpes zoster, caused by varicella-zoster virus (VZ) in the treated lymphoma patients, for example Hodgkin's disease, is a recurrent and sometimes life-threatening occurrence. The incidence of herpes zoster in this particular patient group has been found to be higher than that among patients with solid tumors (3). The immunologic characteristics of Hodgkin's disease which may predispose to VZ infection include loss of pre-existing delayed hypersensitivity by skin testing as well as a loss of ability to acquire new hypersensitivities. Lymphopenia is present, but antibody formation is impaired only in advanced stages, beyond the period when zoster is most frequent. Among the patients with zoster, a significant number will develop disseminated disease superimposed upon the initial segmented lesion associated characteristically with a particular dermatome. Therefore, two specific problems probably related to deficient cell mediated immunity exist in this patient population: first, an increased risk of initial VZ disease and second, with disease occurrence, an increased frequency of dissemination.

Studies in our laboratory have focused on two parameters of cell-mediated immune function: lymphocyte transformation and interferon in responses to specific herpesviral antigens. Our studies have shown that lymphocyte transformation in this system represents a monocyte-dependent T-lymphocyte response, while B-lymphocytes, with the help of monocytes, produce interferon (4). T-lymphocyte transformation is immune-specific, in that only lymphocytes from individuals with serologic evidence of prior exposure will respond *in vitro* with increased DNA synthesis. B-lymphocyte interferon is augmented by herpesvirus disease, but can also be produced by lymphocytes from nonimmune individuals (5). Because of the recognized defect in cell-mediated immunity in patients with Hodgkin's disease, we used these two parameters of immunocompetent cell function to assess specific responses *in vitro* to VZ antigen.

In vitro lymphocyte responses to VZ antigen in lymphoma patients, untreated or in remission, were compared with those of normal individuals. The untreated lymphoma patients showed significantly lower responses than other populations tested, including normals with histories of chickenpox or recent zoster and lymphoma patients in remission. The lymphocyte interferon response was found to be lower in both untreated and lymphoma patients in remission as compared to normals (6). These defects were found to be specific for VZ, since responses in both lymphoma patient groups to herpes simplex antigen were comparable to those of normals. Prospective studies in 86 lymphoma patients analyzing the effect of the first 16 month's therapy upon lymphocyte functions to specific herpes viruses showed that more than 50% of the cases of zoster occurred in patients treated with both radiation and chemotherapy. The increased frequency in this group was accompanied by a specific defect in lymphocyte transformation to VZ antigen which persisted during the observation period (7). These studies also showed that the responses to nonspecific mitogens and T-cell numbers recovered to the range of normal individuals before responses to specific herpes viral antigens.

In the cardiac transplant population, CMV infections occur almost universally with varying manifestations. Some of these clinical findings include unexplained fever, pneumonitis, hepatitis, leukopenia and retinitis in the late period after transplant. The presence of antibody may not protect from shedding virus in throat or urine for prolonged periods or from destructive pathologic lesions. In cardiac transplant patients, defects in both lymphocyte transformation and interferon are present during the period of greatest risk of CMV, a finding similar to that for herpes zoster susceptibility in the lymphoma patients (8).

New forms of herpes zoster have been recently described in which unusually prolonged zoster was observed in patients treated with high doses of immunosuppressive chemotherapy (9). Usually a self-limiting disease, cases of zoster persisted in this group for as long as 6 months. Reduction of immunosuppressive therapy appeared to initiate resolution of zoster lesions and halt dissemination. Mechanism of this prolonged form of herpes zoster may relate to depressed cell mediated immunity in these hosts, since depression in T-lymphocytes was a goal of therapy in the patients studied. These studies suggest that small reductions or short term withholding of immunosuppressive drugs may be sufficient to allow resolution of chronic lesions.

Despite the obvious clinical value of immunosuppression, the use of such drugs must be evaluated both in terms of positive benefits and negative effects. Better understanding of specific immunosuppressive effects will allow their use with minimal adverse consequences.

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Environmental Health Perspectives
Vol. 43, pp. 27-29, 1982

Methods and Approaches for Assessing Immunotoxicity: An Overview

by Jack H. Dean*, Michael I. Luster* and Gary A. Boorman*

The goals of the National Toxicology Program as they relate to immunological evaluation in toxicity assessment are discussed. The advantages of immune function assays for defining cellular injury as subtoxic levels following exposure to general or immunocyte specific chemical toxicants are proposed. A comprehensive screening panel of immune function and host resistance assays is presented in the context of an NIEHS approach for immunotoxicity assessment and methods selection. A second panel for defining the mechanism underlying immunological injury was also described. Studies utilizing these methods and approaches are described in companion papers by our group.

Introduction

A major objective of the National Toxicology Program (NTP) and a major effort at NIEHS is to examine a variety of *in vivo* and *in vitro* approaches to assess toxicity as well as newer research disciplines for evaluating chemicals of environmental concern. Immunology represents one of the disciplines that the NTP is employing to examine possible target organ toxicity as well as generalized toxicity at the cellular and molecular level. Furthermore, the effects of chemicals on host defense to bacterial, viral, fungal and parasitic diseases as well as the surveillance and destruction of neoplastically transformed cells are simultaneously being evaluated. It is our goal at NIEHS to apply systematically the disciplines of immunology and microbiology in a comprehensive manner to study immunotoxicity and altered host resistance and to define a panel of sensitive assays to screen for chemical-induced changes in immunological and host resistance parameters. Extensive studies conducted at NIEHS have suggested that the route and time of exposure relative to the maturational development of the immune system should be of paramount consideration in the design of an evaluation protocol. Several studies have demonstrated that pre- and postnatal exposure to certain chemicals (e.g., TCDD or DES) during the maturation of the thymus-dependent elements of the immune system often produces more profound immunological effects than adult exposure (*1*) or produce long-lasting effects throughout adult life (*2*). Thus, the focus of the discipline of immunology on the evaluation of chemical toxicities may provide beneficial information to facilitate better human risk assessment.

Preliminary data indicate that examination of immune function will add an extra level of sensitivity and safety for defining toxicity at the cellular level. The increased sensitivity of the immune system for detecting cellular toxicity may result from its rapid proliferation or the dynamic and highly regulated nature of its cellular components. This concept is supported by human clinical chemotherapy studies in which cells of the reticuloendothelial system (RES) are among the most frequent target cells for chemical injury. The functional integrity of cell-mediated immunity (CMI) or the quantitation of bone marrow progenitor elements following chemotherapy is a popular means of regulating drug dosage.

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Comprehensive Panel for Evaluating the Immunobiologic Effects

A test for evaluating immunologic effects should meet several criteria if it is to be considered useful and definitive. The test should result in data which can be extrapolated to the human experience and adaptable to practical considerations such as expense, simplicity, time required for completion, reproducibility, uniformity and application to routine subchronic or chronic toxicology studies. Certain compromises are associated with the development of screening techniques. Ideally, immunologic assays that will identify and assess the risk potential of a chemical or drug are desirable; however, in general, no single assay can accomplish this task, and as such, a panel of selected assays that have been validated in experimental rodent models and human clinical studies is recommended.

Currently, a wide variety of assays is being utilized in the Immunology Program at the National Institute of Environmental Health Sciences to assess immunologic and host resistance alterations following chemical exposure (Table 1). This is not to suggest that it is necessary or even desirable for evaluation laboratories to employ such a comprehensive assay panel. Rather, at our present stage of knowledge, it is reasonable that a few laboratories perform a comprehensive panel of assays concurrently to determine the relative simplicity, reproducibility and predictability of these methods for detecting altered immunological and host resistance functions. Some of these methods will undoubtably be selected to screen new chemicals and drugs for immunotoxicological potential. The detailed methodologies of these assays as performed at NIEHS have been described elsewhere in detail (*3*). These procedures were selected because they are reproducible and easily standardized. During the method selection phase of our research, major emphasis has been placed on assays that could be automated, routinized or require only microquantitates of cells or body fluids. These procedures involve the evaluation of pathotoxicology indicators, host resistance models, delayed-type hypersensitivity, cell-mediated immu-

Table 1. Comprehensive screening panel for defining immune alterations currently being evaluated at NIEHS.

Parameter	Procedure performed
Pathotoxicology	Hematology profile (hemoglobin, red blood cell count, white blood cell count, differential
	Liver chemistries (SGPT, triglycerides, cholesterol)
	Serum proteins (albumin, globulin, A/G, total proteins)
	Weights (body, spleen, thymus, liver, kidney, heart, lung, brain)
	Histology (liver, thymus, lung, kidney, spleen)
Host resistance	Tumor assays (tumor cell challenge $TD_{10\text{-}20}$ and radiometric tumor mass)
	Listeria monocytogenes $LD_{10\text{-}20}$ challenge
	Endotoxin hypersensitivity-$LD_{10\text{-}20}$
	Expulsion of *Trichinella spiralis*
Delayed hypersensitivity	Radiometric assay with T-cell-dependent antigen
Lymphocyte proliferation	One-way mixed leukocyte culture
	Mitogens (PHA, Con A, LPS)
Humoral immunity	Immunoglobulin levels (IgG, IgM, IgA)
	Antibody response to T-dependent and T-independent antigens
Macrophage function	Resident peritoneal cell numbers and nonspecific esterase staining
	Phagocytosis of ^{51}Cr-SRBC
	Lysosomal enzymes (5′-nucleotidase, acid phosphatase, leucine amino peptidase)
	Cytostasis of tumor target cells
	Cytolysis of tumor target cells
	RES clearance using ^{125}I-trioleion
Bone marrow colony forming units	CFU-S (multipotent, hematopoietic stem cells)
	CFU-GM (granulocyte/macrophage progenitor)
	CFU-E (erythrocytes progenitor)
	Cellularity
	^{59}Iron incorporation in bone marrow

Table 2. Procedures to define the mechanism of chemically induced immune alterations.

Altered parameter	Further procedures to perform
Pathotoxicology	Bioaccumulation study Hormone levels Pair-feeding
Host resistance	Virus challenge Staphylococcus or Streptococcus challenge (B-cell-dependent)
Cell-mediated immunity	DHR using T-cell independent antigen Suppressor cell studies with mitogens and cocultures Helper cell studies Spontaneous cytotoxicity Lymphokine production
Antibody-mediated immunity	Mishell-Dutton assay and cocultures Local production of antibody Titer of serum antibody–T-cell-independent antigen
Bone marrow toxicity	Serum levels of colony stimulating factor (CSF) CSF and prostaglandin synthesis by macrophages and bone marrow stromal cells

nity, humoral-mediated immunity, macrophage function and bone marrow progenitor cells. Attempts are being made to correlate changes in immune function with altered host resistance.

If the *in vivo* and *in vitro* data obtained from such carefully planned studies using these screening procedures are negative, there can be reasonable confidence in the safety of the drug or chemical under the conditions and dosages defined.

A major limitation in risk assessment has involved extrapolation of dose response curves from effect to no-effect levels or from rodent model systems to humans. However, if conservative extrapolations are made by using data from appropriate assays, the most relevant and accurate estimate possible will be obtained. At present the methods listed are in the validation and selection stage for later application of some of these to routine toxicity assessment when immunology studies are indicated.

If warranted by preliminary data, additional tests can be used to examine the mechanisms by which a particular chemical or drug alters immune function (Table 2). These assays can provide additional information regarding mechanisms of immunotoxicity and may provide a means by which to circumvent or abolish the undesired effects of the agent. If the pathophysiologic mechanism responsible for the effect or the target cell is defined, possibly new analogs of the chemical or drug can be synthesized which produce the desirable effects but not the undesirable ones (e.g., synthetic penicillin).

The following papers in this session describe, in detail, methods and approaches utilized by leading laboratories in this rapidly developing field.

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Environmental Health Perspectives
Vol. 43, pp. 31-36, 1982

Cell-Mediated Immunity and Its Application in Toxicology

by Michael I. Luster,* Jack H. Dean† and Gary A. Boorman†

A variety of *in vivo* and more recently *in vitro* assays have been described to assess cell mediated immunity (CMI). Two methods routinely employed in our laboratory to assess CMI following exposure to chemicals in rodents include delayed hypersensitivity and *in vitro* lymphoproliferation. Preliminary studies indicate that depressed delayed hypersensitivity responses, as performed by a radiometric assay, correlates with altered susceptibility to infectious agents and tumor cell challenge following exposure to immunotoxic chemicals. Furthermore, suppression of T-cell lymphoproliferative responses to at least 50% below control values correlated with depressed delayed hypersensitivity responses and altered host susceptibility. On the other hand, when suppression of T-cell lymphoproliferative responses are within 50% of control values, delayed hypersensitivity and host susceptibility parameters are not affected. Assuming adequate technical expertise and accurate data interpretation, CMI assays of these types can provide a valuable data base for toxicology studies and immunotoxicity assessment.

Introduction

Reports indicating that exposure to various environmental pollutants leads to immunological alterations in laboratory animals and even man has raised concerns regarding sensitive methodology for routine assessment of immunotoxicity (*1*, *2*). One concern in assay battery development is the use of appropriate models to examine cell-mediated immunity (CMI). In recent years, large numbers of *in vivo* and more recently *in vitro* assays have been developed to assess CMI. To insure accurate evaluation an *in vivo* assay, which represents a holistic approach, and a more defined *in vitro* assay for CMI should be employed. Routinely, CMI is assessed in our laboratory by quantitating *in vivo* delayed hypersensitivity responses using a radiometric technique and *in vitro* lymphoproliferative responses following chemical exposure (*3*). These techniques and their relationship to other aspects of immunity (i.e., host susceptibility, humoral immunity and the reticuloendothelial system) are examined. Considerations in selecting appropriate CMI assays include sensitivity, simplicity and extrapolation of dose-response curves from effect to no-effect levels as well as from rodent model systems to humans.

Assays for Cell-Mediated Immunity

CMI is evoked by T-lymphocytes, although macrophages and to a lesser extent polymorphonuclear leukocytes (PMNs) play an active role in these reactions (*4*). CMI is responsible for classical delayed hypersensitivity reactions (DHRs), rejec-

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Table 1. Assays commonly employed to assess cell mediated immunity.

- *In vivo*
  - Delayed hypersensitivity
  - Skin grafts
  - Graft vs. host
- *In vitro*
  - Lymphocyte proliferation
    - Mitogens
    - Specific antigens
    - Mixed lymphocytes
  - Enumeration of lymphocyte subpopulations
    - Antigenic determinants on surface membrane
    - Rosette formation
    - Complement and Fc receptors
  - Mediator production by lymphocytes
    - Migration inhibitory factors
    - Lymphocytotoxicity
    - Immune interferon
  - Assays for polymorphonuclear leukocytes and macrophages
    - Phagocytosis
    - Chemotaxis
    - Cytostasis
    - Chemiluminescence

tion of transplants, tumor immunity, some autoimmune diseases and resistance to facultative intracellular parasites as well as many recurrent infections. In general, *in vivo* assays predominantly measure the effector mechanism of CMI, while *in vitro* assays may evaluate cellular components. A number of these assays appear to represent a sensitive and reliable indicator of immunotoxicity and will be discussed in some detail.

Table 1 lists some of the more common techniques employed to assess CMI in both humans and laboratory animals and have been discussed in detail elsewhere (*5*). These assays are, in general, fairly quantitative and capable of detecting either depression or augmentation. Despite the recent development of *in vitro* techniques, delayed hypersensitivity testing remains the most widely accepted means of assessing CMI. Other *in vivo* measures that have been used to assess CMI in laboratory animals, include graft vs host and skin graft assays (*2*). More recently, a number of *in vitro* assays have been developed to assess CMI (*5*). The microculture lymphocyte proliferation (LP) assay utilizes T- and B-cell mitogens (usually plant lectins or bacterial products), specific antigens or alloantigens in a one-way mixed leukocyte culture (MLC) to examine proliferative function in lymphocyte populations (*6*). The assay provides a relatively simple, reproducible and semiquantative *in vitro* correlate for CMI. Various methods have been used to enumerate lymphocyte subpopulations in peripheral blood of humans and spleens of mice (*7*). Enumeration, alone, however, does not measure functional capabilities. B-lymphocytes possess readily detectable membrane immunoglobulins (mIgs) on the cell surface which can be detected using fluorescein-conjugated antiserum to the Igs. In addition, B-cells possess receptors for complement and the Fc region of IgG although other cell subpopulations also possess these receptors. T-lymphocytes, on the other hand, can be recognized in some species, excluding rodents, by spontaneous formation of rosettes with sheep erythrocytes (E-rosettes). Additionally, T-lymphocytes can be identified by distinct antigenic markers on the surface membrane (e.g., Thy 1). T-lymphocytes that are antigenically or nonspecifically stimulated, elaborate soluble mediators referred to as lymphokines. The biological function of these lymphokines are measured by a variety of *in vitro* techniques. Migration inhibitory factors retard the migration of macrophages or PMNs. Lymphocytoxicity assays quantitate cytotoxic or cytostatic activity elaborated when sensitized T-cells are cultured with specific soluble or particulate antigen. The end point in this type of assay is quantitation of target cell destruction. Immune interferon, another type of lymphokine, is active against a wide range of viruses. Interferon production is readily quantitated by determining reduction of viral plaques in an appropriate tissue culture system.

There are also a number of *in vitro* techniques that specifically assess macrophage and PMN functions. Those assays routinely employed in our laboratory include chemotaxis, phagocytosis, cytostasis, and chemiluminescence (*8*). Chemotaxis reflects the ability of various cells including PMNs, monocytes and/or lymphocytes to preferentially migrate to an increasing chemical gradient. These chemical attractants include components of complement and bacterial products. Phagocytosis of ^{51}Cr-radiolabeled SRBCs is determined on resident and induced adherent peritoneal cells. Adherent cells are also examined for their ability to inhibit the growth of various tumor cells in a microculture growth inhibition assay referred to as cytostasis and is presumably a measure of activation.

Delayed Hypersensitivity Responses

Delayed hypersensitivity responses (DHRs) remain the most widely accepted means of clinical or experimental assessment of CMI. The reaction is initiated by actively sensitized T-lymphocytes which respond specifically to the antigen through the

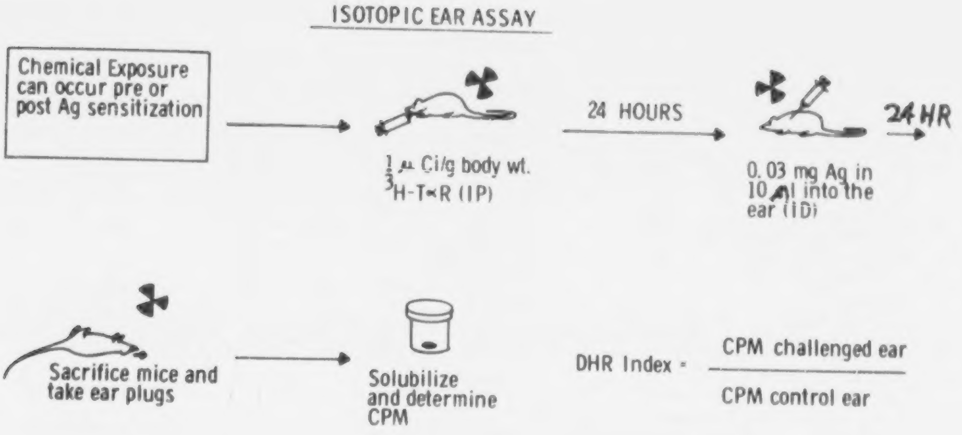
ISOTOPIC EAR ASSAY
Chemical Exposure can occur pre or post Ag sensitization
24 HOURS
24 HR
1 µ Ci/g body wt. ³H-T*R (IP)
0.03 mg Ag in 10 µl into the ear (ID)
Sacrifice mice and take ear plugs
Solubilize and determine CPM
DHR Index = CPM challenged ear / CPM control ear

FIGURE 1. Radiometric ear assay for determining DHRs in chemically exposed mice. Mice are sensitized to an antigen, (e.g., 100 µg keyhole lymphet hemocyanin in Freund's adjuvant). Ten or more days following the last sensitization, mice received 1 µCi/g body weight of H-TdR; 24 hr later, animals are given 30 µg KLH in a volume of 10 µl into the pinna of one ear. The other ear serves as a control and is injected with saline. After 24 hr the mice are sacrificed, ear plugs taken, solubilized and radioactivity determined. The data are expressed as an index (CPM Challenged Ear/CPM Control Ear).

release of lymphokines and/or development of specific cytotoxicity without the participation of antibody (*9*). Locally it is manifested by cellular infiltration at the site of antigen administration. In humans, DHRs are measured by the intradermal administration of a panel of antigens to which previous exposure is likely (*10*). The response is determined at the challenge site by measuring the diameter of erythema and induration at 24 and 48 hr. Anergy (reduced DHRs) can be interpreted as positive response to one or less antigens and/or reduced skin reactions. Depressed DHRs, as most recently demonstrated by MacLean (*11*), correlate with decreased host resistance to infectious agents. In these studies 178 patients were serially skin tested following surgery. Of the 135 patients in this study who displayed normal DHRs only 21% developed sepsis and 2% deaths. In contrast, 65% sepsis and 7% mortality occurred in patients demonstrating anergy.

Radiometric assays, which offer greater sensitivity than skin tests, are often employed to assess DHRs in rodents (*12, 13*). We routinely employ a modification (*3*) of a radiometric ear assay originally described by Lefford (*13*). This method, as performed in our laboratory, is depicted in Figure 1. Administration of tritiated thymidine (^{3}H-TdR) prior to antigenic challenge radiolabels monocyte precursors in the bone marrow. The response is presumably represented by mononuclear infiltration around the reaction site.

In Table 2 the effects of chemical exposure on DHRs, as measured by the radiometric ear assay, are compared to alterations in antibody responses and host resistance. The panel of host resistance assays represented in Table 2 are considered under the influence of CMI and are discussed in detail elsewhere (*14*). Although preliminary, the data in Table 2 indicate that decreased DHRs correlate with increased host susceptibility to artificial infection. DHRs did not correlate with antibody production, since exposure to benzo(a)pyrene induced severe depression of antibody production without concomitant effects on DHRs. As would be expected, benzo(a)pyrene failed to alter hose resistance. In mice, we routinely employ keyhold lymphet hemocyanin (Pacific Biomarine, Pacific Grove, Calif.) in Freund's adjuvant as an antigen although a variety of sensitizing antigens are suitable including extracts of *T. spiralis*. Good responses can be obtained in rats sensitized with complete Freund's adjuvant (e.g., H37Ra; Difco) and subsequently challenged with purified protein derivative (PPD; Parke-Davis) (*15*). While a variety of antigens can be used to study DHRs, one needs to select an antigen and sensitization schedule that favors CMI and not antibody synthesis. Furthermore, consideration must be given to the time of sensitization in relationship to chemical exposure. Sensitization prior to chemical exposure may be considered an assay for memory cell function, while short-term immunological alterations

Table 2. Relationship of DHRs, antibody responses and host susceptibility in mice following exposure to various chemicals.

Species	Chemical treatments[a]	Total dosage, mg/kg	Antigen[b]	DHR index	Antibody responses[c]	Host resistance[c]
Mice	Mpme	–	KLH	2.93 ± 0.28	–	–
	DES (pre-sens)	8	KLH	2.94 ± 0.33	↓	↓ ↓
	DES (post-sens)	8	KLH	1.88 ± 0.15[d]	↓	↓ ↓
	Cyclophosphamide	180	KLH	1.54 ± 0.11[d]	↓ ↓	↓ ↓
	TCPP	100	KLH	3.13 ± 0.26	–	–
	Benzo(a)pyrene	400	KLH	2.73 ± 0.19	↓ ↓	–
	None	–	*T. spiralis*	3.23 ± 0.28	–	–
	DES (pcst)	8	*T. spiralis*	1.67 ± 0.23[d]	↓	↓ ↓
Rats	None	–	PPD	5.64 ± 0.65	–	–
	TCDD	0.02	PPD	3.17 ± 0.44[d]	–	↓

[a] All mice were exposed to the various chemicals subchronically at 6-8 weeks of age. Rats were treated pre/postnatally (via maternal exposure) as described previously (*15*).

[b] Abbreviations: DES = diethylstilbestrol; TCPP = tris(1,3-dichloropropyl) phosphate; TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin; KLH = keyhole lymphet hemocyanin; *T. spiralis* = an antigen extract.

[c] Code: — = no effect; ↓ = slight to moderate; ↓ ↓ = marked effect.

[d] Significant decreased from control values ($p < 0.01$).

may be missed if sensitization is not initiated until chemical exposure has been completed. Of course, subchronic 30 to 90 day toxicity studies will tend to minimize these types of problems.

Lymphoproliferative Responses

Lymphoproliferative (LP) responses are a widely used correlate of CMI and can be defective in the absence of lymphopenia. In the microculture LP assay, general mitogens (e.g., plant lectins, bacterial products), specific antigens or alloantigens (i.e., mixed lymphocytes) are used to stimulate selectively the cell proliferation of splenic or peripheral lymphocytes (*6*). Proliferation is measured by ^{3}H-TdR incorporation into DNA, represented as CPM. Unlike specific antigens, mitogens are polyclonal activators some of which are capable of tions (see Table 3). In mice, phytohemagglutinin (PHA) and concanavalin A (Con A) are often used to activate T-lymphocytes, while lipopolysaccharide (LPS), isolated from gram-negative bacteria, is used to transform B-lymphocytes (*3*). Depressed LP responses occur in humans with inherited immunodeficiency (primary) diseases as well as various pathological or physiological conditions, including malignancies (Hodgkin's disease, lung cancer), intestinal lymphangiectasia, aging, chronic lymphatic leukemia, nutritional deprivation and in severe stress (e.g., post-surgery) (*6*, *17*).

Depressed LP response in experimental animals with normal numbers of lymphocytes has usually been interpreted as failure of cell activation. Recent studies have indicated that this may occur through tolerance or suppressor substances produced by regulatory subpopulations of macrophages and T-lymphocytes (*6*, *7*). Other factors, however,

Table 3. Lymphocyte activators (mitogens) and their characteristics.[a]

Source	Common examples	Cells transformed
Plant extracts	Phytohemagglutinin	T
	Concanavalin A	T
	Pokeweed mitogen	T or B
Bacterial products	Lipopolysaccharide (Lipid A)	B
	Staph. enterotoxin	T
	Purified protein derivative	B
Antibody reagents	Anti-immunoglobulin	B
	CHO antibodies	T or B
Miscellaneous chemicals	Phorbol ester	T
	Dextran sulfate	B
	Metal ions (zinc, mercury, lead, etc.)	T or B

[a] Modified from Cunningham et al. (*16*).

Table 4. Relationship of lymphoproliferative responses to DHRs, antibody PFCs and host susceptibility, following chemical exposure in mice.

Chemical treatment[a]	Total dosage, mg/kg	Deviation from control, %: T-Cell mitogen	B-Cell mitogen	DHR	Antibody PFC's	Host resistance[b]
DES	8	70 ↓	29 ↓	33 ↓	23 ↓	↓ ↓
Cyclophosphamide	180	97 ↓	97 ↓	47 ↓	99 ↓	↓ ↓
TCPP	100	25 ↓	32 ↓	6 ↑	13 ↑	–/↓
Benzo(a)pyrene	400	32 ↓	54 ↓	4 ↓	75 ↓	–
TCDD	0.02	53 ↓	18 ↓	60 ↓	ND	↓
PBB	1220	39 ↓	35 ↓	12 ↑	5 ↑	–
o-Phenylphenol	2000	7 ↓	11 ↓	13 ↑	13 ↑	–

[a] TCDD was administered in mice pre/postnatally (via maternal exposure) as described previously (*18*). PBB was given chronically (*19*). All other chemicals were administered as described in Table 2.

[b] Code:— = no effect; ↓ = slight to moderate; ↓ ↓ = marked effect: ND = not done.

should be considered as possibly causing depression of LP responses. Some of these factors include: a chemically induced lymphocytotoxicity as may occur with TCDD (*1*); redistribution of lymphocyte subpopulations (i.e., B-, T- or null cells) as may occur in chronic lymphatic leukemia; and/or an early maturational defect in lymphocyte development since mature lymphocytes respond differently to mitogens than less mature lymphocytes. This is not to say that LP assays are not a reliable predictor of immune alteration but, rather, may prove to be an extremely sensitive indicator of immunotoxicity, if appropriately interpreted.

Table 4 summarizes some recent studies in our laboratories in which LP responses were compared to a variety of immunological and host susceptibility assays in mice following exposure to therapeutic and environmental chemicals. All data reported were obtained in animals administered dosage levels that did not induce overt signs of toxicity. As in Table 2, resistance to the biological agents used to assess altered host susceptibility in these studies are considered primarily under the influence of CMI and not humoral mediated immunity (HMI). While preliminary, certain profiles emerged following examination of these data. As would be expected a large number of chemicals examined (e.g., *o*-phenylphenol) did not effect any host resistance or immune function assays. Other chemicals, such as cyclophosphamide, diethylstilbestrol and 2,3,7,8-TCDD, were clearly immunotoxic, causing greater than 50% depression of LP responses, suppressed DHRs, increased host susceptibility and in most cases depressed antibody PFC responses. In earlier studies these chemicals have been shown to be selectively toxic to the immune system (*1*, *8*). Another group of chemicals, such as tris(1,3-dichloropropyl) phosphate (TCPP) and polybrominated biphenyls (PBB), displayed minimal suppression of LP responses, ranging from 25 to 50% below control values, without concomitant effects on other immune parameters or host susceptibility. Although presently speculative, this immune profile may be representative of a general toxicity rather than a selective effect on the immune system. Support for this hypothesis has been provided in collaborative studies with Boorman (*20*). In these studies, mice with depressed LP responses following exposure to TCPP or PBB also revealed decreased hematopoietic stem cell proliferation using *in vitro* spleen and bone marrow colony forming assays. Since these chemicals are biologically active, probably capable of binding to DNA, it would be expected that the more proliferative cell populations (i.e., stem and lymphoid cells) would have a greater predilection to chemical injury. Benzo(a)pyrene appeared selectively to affect B-lymphocytes while sparing CMI, although T-cell LP responses were somewhat affected. This is not a unique phenomenon in immunotoxicology, since exposure to heavy metals under many circumstances induces similar effects (*21*).

In summary, many assays are available to assess CMI following chemical exposure. Since, in general, these assays represent "biological phenomena" one should employ both *in vivo* and *in vitro* assays for proper assessment. Assay selection should be based upon sensitivity, simplicity and extrapolation of dose response curves from effect to no-effect levels as well as from rodent systems to humans. Preliminary studies reported here, which should be interpreted with caution, suggest that a depressed DHR corresponds with increased host susceptibility. On the other hand, while marked suppression of T-cell LP responses correlates with suppressed DHRs, less severe depression does not correlate with other immune or host resistance parameters, but rather may be an early indicator of general toxicity.

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Environmental Health Perspectives
Vol. 43, pp. 37-39, 1982

In Vitro Assessment of Humoral Immunity Following Exposure to Heavy Metals

by Loren D. Koller*

The immune system of animals and man is extremely complex. This report will discuss the effect metals have on one segment of the immune system; that is, humoral immunity. Humoral immunity is essentially the production of antibody in response to an antigen. The B-lymphocyte is the primary cell responsible for producing antibody. However, this cell is regulated by T-lymphocytes and macrophages.

Many methods are available to assess humoral immune responses. A multitude of immunoassays have been developed for enumeration of serum antibody. Some of these are immunodiffusion, complement fixation, serum neutralization, hemagglutination, radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). Detection of antibody-forming cells distinguishes between effect on antibody production compared to degradation of preformed antibody. Other available methods are measurement of surface receptor (Fc and complement) activity on B-cells. Mitogens (T-independent) have also been regarded as a measurement for humoral immunity.

In our laboratory, we have developed an ELISA technique that is appropriate for general assessment of humoral immune responses resulting from chemical exposure. This technique is highly sensitive and can be mechanized. The assay can easily be incorporated into the drug and chemical efficacy testing programs of industry.

The immune system of animals and man is complex and cooperation between the three major components, humoral, cell-mediated and macrophage systems, is often necessary for optimal expression. The major identifying property of humoral immunity is the production and secretion of antibody by lymphocytes. The B-lymphocyte is th[illegible] directly responsible for humoral immunity by differentiation into antibody producing (plasma) cells after antigen stimulation.

Introduction of antigen into a host results in either direct stimulation of B-cells or the antigen may initially be phagocytized by macrophages which transfers the antigenic determinants to B-lymphocytes. The sensitized small lymphocytes progress through a series of transformations into large lymphocytes and finally lymphoblasts, which differentiate into antibody-secreting plasma cells or small memory lymphocytes. The reaction commences when antigen molecules encounter small lymphocytes with surface antibody to that particular antigen. The antigen is bound to the antibody which provokes the small lymphocytes to divide and differentiate. During differentiation, the surface antigen–antibody complexes are lost and replaced by new antibody receptors. The memory cells possess surface immunoglobulin receptors similar to the original small lymphocytes while plasma cells synthesize and secrete circulating antibody.

The memory cell, on the other hand, is a resting antigen-sensitized B-cell which responds to a second exposure of that antigen by rapid production of large amounts of antibody. These antibodies usually have a greater affinity and specificity for antigen than those produced after initial exposure.

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Antibody responses to many antigens require cooperation between at least two types of lymphocytes for optimal expression. Differentiation of bone marrow-derived B-cells into antibody-secreting plasma cells, is usually regulated by thymus-derived T-cells. The function of the T-cell and its soluble factors is not to produce antibody, but rather to aid in activation and proliferation of B-cells (*1*). A third cell type, the macrophage, also has an important role as an accessory cell by cooperating with T-cells and aiding the response of B-cells to antigens.

There are at least four types of T-cells recognized to date. These include cytotoxic, helper, amplifier and suppressor T-cells. All but the cytotoxic lymphocyte function in regulating the humoral immune response. The helper T-cell is required for activation of B-cells to many antigens, while the amplifier T-cell regulates proliferation of activated B-lymphocytes. This cooperation may occur either by cell to cell contact or by release of soluble factors by T-cells (*2*). The other T-cell, suppressor T, is responsible for suppressing antibody synthesis by inhibiting B-lymphocytes. In general, B-cell activity is regulated by a balance of helper and suppressor T-cells. Exposure to a weak or small antigen dose may induce helper T-cells, while a strong antigen or larger dose may induce suppressor T-cells, thereby controlling amount of antibody synthesized.

Macrophages are also modulators of humoral immunity. Macrophages, like B-lymphocytes, have receptors for complement and the Fc portion of immunoglobulin. One function of macrophages is to localize antigen and bring it closer to an antibody-forming lymphocyte. Macrophages may also process antigen in lysosomes making it highly antigenic (*3-5*). Like the T–B cell interaction, this cooperation may occur by physical contact between the macrophage and B-cell or by soluble factors released from the macrophage. Macrophages also activate T-lymphocytes to produce nonspecific helper activity for antibody production (*6*, *7*).

In summary, the pathway of the humoral immune system from antigen stimulation to antibody synthesis is extremely complex. The B-lymphocyte is the progenitor of antibody-forming plasma cells, but optimum responses are not usually achieved without participation by T-lymphocytes and macrophages. Furthermore, it is a balance between helper T and suppressor T as well as amplifier T and macrophages that regulate B-cell activity and consequently the humoral immune response.

Immunoassays used to examine humoral immunity include those which measure antibody titers (immunodiffusion, complement fixation, serum neutralization, hemagglutination, passive hemagglutination, radioimmunoassay, enzyme-linked immunosorbent assay, etc.), antibody synthesis (Jerne plaque) and B-lymphocyte receptors (EA = Fc and EAC = C3). Humoral immunity may be determined after initial exposure to the antigen (primary response) or after rechallenge with antigen (secondary or memory response). Staining of surface immunoglobulin will identify B-lymphocytes and enumerate percent B-cells. Many antigens, both T-dependent and T-independent, have been utilized in these investigations. Some of these antigens are tetanus toxoid, sheep red blood cells, bovine serum albumin, bovine gammaglobulin, *Salmonella typhi*, pseudorabies virus, influenza virus, keyhold limpet hemocyanin and lipopolysaccharide.

Metals and metal compounds responsible for reduced circulating antibody titers are lead (*8*, *9*), cadmium (*9*, *10*), methylmercury (*9*, *11*, *12*), arsenic (*12*), nickel (*13*), chromium (*13*), and platinum (*14*). Antibody synthesis has been inhibited by lead (*12*, *15-17*), cadmium (*17*, *18-20*), methylmercury (*12*, *21*, *22*), zinc (*23*), arsenic (*12*), silica (*24*) and magnesium (*25*). Selenium, on the other hand, has stimulated both antibody synthesis and circulating antibody titers (*26-28*). Lead and cadmium (*29*) inhibit the activity of B-lymphocyte receptors, while methylmercury (*30*) actually augments the complement receptor activity on B-cells. Finally, lead, cadmium and methylmercury have been demonstrated to affect immunological memory (*31*).

It has been well documented that many of these metals compromise the immune system of experimental animals. Damage may occur to a particular cell (B, T or macrophage) or may involve more than one cell which regulates the proliferation and differentiation of other cells responsible for normal function of the immune system. We have accepted the ELISA as the test procedure of choice in our laboratory for humoral immunity. This assay is simple to perform, reliable, highly sensitive, economically feasible, has minimum variation, requires only a few test animals and can be mechanized. Further, in all of our investigations of environmental chemicals in which antibody titers were decreased, antibody synthesis was also inhibited: an indication that the more complex antibody synthesis techniques are unnecessary to perform except for special instances. Therefore, we are confident that the ELISA technique is appropriate for general assessment of humoral immune responses in animals that have been exposed to chemicals.

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Environmental Health Perspectives
Vol. 43, pp. 41-52, 1982

In Vivo Assessment of Immunotoxicity

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The organs, tissues, and cells of the lymphoreticular system have received considerable attention as targets for chemicals causing adverse effects. A basic toxicological approach is described for assessing the risk of a chemical perturbing the immune system. CD-1 mice were exposed for 14 or 90 days to one of several chlorinated hydrocarbons: 1,2-dichloroethane, 1,2-dichloroethylene or 1,1,2-trichloroethylene. Other mice were exposed to dexamethasone, a known immunosuppressive agent. The immune system is evaluated against a background of the more standard toxicological parameters such as fluid consumption, body and organ weights, hematology, clinical chemistries, and blood coagulation. Reported here are the results for the male mice after 14-day exposure to three chlorinated hydrocarbons and after 90-day exposure to 1,2-dichloroethane and dexamethasone.

Acute toxicity studies were performed to provide a basis for doses used in the subchronic studies. The LD_{50} values are reported.

The status of the humoral immune system was determined by measuring the number of IgM spleen antibody-forming cells to sRBC, the serum antibody level to sRBC, and the lymphocyte response to the B-cell mitogen, LPS. Of the three chlorinated hydrocarbons, only dichloroethane produced a significant ($p < 0.05$) reduction in antibody-forming cells. The other two chemicals produced trends towards suppression. Mice exposed to dichloroethane in the drinking water for 90 days showed no alteration in AFC, serum antibody titers or response to the B-lymphocyte mitogen, LPS. Subchronic 90-day exposure to dexamethasone produced a dose-dependent inhibition of AFC/spleen but not AFC/10^6 spleen cells when measured on the peak day of response. Response to LPS was not altered, and spleen weight and spleen cell number were reduced as much as 42%. These data suggest that dexamethasone administered in the drinking water is nonspecifically cytotoxic to the spleen cells.

Cell-mediated immunity was assessed by measuring the DTH response to sRBC and the response to the T-lymphocyte mitogen, concanavalin A. After 14 days of exposure, trichloroethylene produced a 15 and 60% suppression at 24 and 240 mg/kg, respectively. Dichloroethylene produced a non-dose-dependent inhibition at 4.9 and 49 mg/kg, which was slight, but significant ($p < 0.05$). Subchronic 90-day exposure to dichloroethane did not alter the DTH response or spleen lymphocyte response to concanavalin A. In contrast, dexamethasone produced a dose-dependent inhibition of the DTH response and a hyperresponsiveness to concanavalin A.

Dichloroethane did not alter the functional activity of the reticuloendothelial system, as measured by the vascular clearance rate and tissue uptake of ^{51}Cr sRBC. In the case of dexamethasone exposure, only the spleen and thymus showed decreased uptake of ^{51}Cr sRBC, which was directly related to decrease in size.

The approaches and results from these types of studies provide a basis for judging a chemical's potential risk to the immune system.

Introduction

The organs, tissues and cells of the lymphoreticular system have received considerable attention as targets for chemicals which cause adverse effects. The immune system, like all other systems in the body, is complex, with several types of cells working independently and in concert to carry out a role in homeostasis. Immunotoxicology is the subject of increased activity and awareness because the physiology and biochemistry of the immune system and the basic processes involved in host defense mechanisms are now better understood. Another reason for this interest is that the immune system's cells, having been exposed to a chemical, can be readily removed and their function(s) examined *in vitro*. This is in keeping with the direction that toxicology is taking—complementing morphological changes with functional alterations.

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We are currently using a basic toxicological approach to assess a chemical's risk to the immune system. This approach includes using male and female random-bred mice in subchronic 14-day exposures as range-finding studies, followed by a more definitive subchronic 90-day study. The route of administration for these studies mimics human exposure. Except for carcinogenic, mutagenic and reproductive effects, the subchronic 90-day exposure is believed to produce an adequate manifestation of a chemical's toxicity. This may not prove true for the immune system because of long-lived T-lymphocytes. The immune response is evaluated by using assays that measure complex cell interactions, interactions of intermediate complexity and certain direct cellular assays. The analysis of the immune system is determined against a background of more conventional toxicological procedures such as body and organ weights, hematology, clinical chemistries, urinalysis and microsomal enzymes.

The data presented in this paper are selected from results obtained from acute, subchronic 14-day studies on dichloroethane, dichloroethylene and trichloroethylene and subchronic 90-day studies on dichloroethane and dexamethasone. Presented are results only from male mice. The results of the female mice were not remarkably different from the males. Dichloroethane was selected because of its environmental importance and because it was devoid of effects on the immune system. Dexamethasone was used because of its known immunosuppressive effects and solubility in water. The chemicals were administered orally: by gavage for the 14-day exposure and in the drinking water for the 90-day exposure. Historical controls are shown to provide indicators of variance for these immunologic parameters in random-bred mice.

Materials and Methods

Animals and Housing

All CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Mass. and housed four per shoebox plastic cage with sawdust bedding and maintained on Agway Lab Chow ad libitum. The animal rooms were maintained at 70-75°F and relative humidity at 40-60%. The light-dark cycle was maintained on 12-hr intervals. Upon arrival, all mice were quarantined for 1 week prior to use.

For acute toxicity studies, 6-week-old male and female mice were used, and for the subchronic 14- and 90-day studies, 4-week-old mice were obtained, with exposures beginning at 5 weeks of age.

Chemicals

1,2-Dichloroethane, Lot #120487, was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin; trans-1,2-dichloroethylene, Lot #083187, was also obtained from Aldrich Chemical Co. 1,1,2-Trichloroethylene, Lot #713839, was obtained from Baker Chemical Co., Phillipsburg, N.J. Dexamethasone phosphate, Lot #86837, was a gift of Merck, Sharpe, and Dohme, Inc., West Point, Pa. Flumethasone pivalate, Lot #R-4088, was supplied through the courtesy of Ciba-Geigy, Summit, N.J.

Chemical Administration

Solutions of dichloroethane, dichloroethylene, trichloroethylene, and dexamethasone were prepared fresh daily for acute and subchronic 14-day studies and the appropriate concentrations were administered by gavage in a volume of 0.01 ml/g body weight to achieve the desired dose. For the subchronic 90-day studies, dichloroethane was administered in the drinking water, diluted in deionized water to concentrations of 0.02, 0.2 and 2.0 mg/ml. For dexamethasone, the concentrations for the 90-day exposure were 0.1, 1.0 and 2.0 μg/ml. Drinking water solutions were maintained at room temperature in amber-colored bottles with stainless steel spouts fitted through the corks. The solutions were changed twice weekly, and less than 10% of dichloroethane and dexamethasone was lost during the three to four days between water bottle changes, as measured by gas chromatography for dichloroethane and high pressure liquid chromatography for dexamethasone.

For the subchronic 90-day study, fluid consumption was estimated by the change in fluid weight over the three- or four-day period when solutions were changed. Twelve cages of control mice and eight cages of each treatment group for each sex were used to estimate fluid consumption. Chemical consumption was calculated from fluid consumption and is reported as ml/mouse/day and mg/kg/day.

Acute Toxicity

The chemicals were administered to male and female mice by an 18-gauge stainless steel stomach tube 1 hr after fasting from food. Following gavage, the mice were observed for overt toxicological effects continuously for 4 hr and then twice daily for 14 days. Mice dying during the experimental procedure were necropsied, and gross pathological changes were described. Mice surviving the 14-day observation period were sacrificed and gross pathology described. Log probit analysis (*1*) was used to determine the LD_{50} and 95% confidence limits.

Animal Necropsy

Animals were anesthetized with chloroform, and blood was collected immediately by cardiac puncture. Gross pathological examinations were performed on all mice. The organs (brain, liver, spleen, lungs, thymus, kidneys and testes) were then removed, trimmed and weighed. Certain organs were used in further studies.

The plasma from the blood samples was assayed for extrinsic activity by measuring the prothrombin time. Reagents for this assay were obtained from General Diagnostics. Fibrinogen levels were determined by the kinetic method, using reagents from Dade Diagnostics, Inc.

Hematology

Blood samples were taken in 3.2% sodium citrate. Leukocyte, erythrocyte and platelet counts were performed on a Coulter Counter, Model ZBI. Hematocrits were performed with microhematocrit equipment, and hemoglobins determined as cyanomethemoglobin.

Humoral Immunity: Spleen Cell Antibody Response

The primary IgM response to sheep erythrocytes was estimated by the hemolytic plaque assay of Jerne [illegible] as modified by Cunningham and Szenberg (*3*). Mice were immunized four or five days prior to sacrifice with 4 × 10^8 sheep erythrocytes by IP injection. Spleen cell suspensions were prepared in RPMI 1640 culture medium by using stainless steel mesh screens and adjusted to a cell concentration of [illegible] for assay of antibody-forming cells (AFC).

As compliment to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Seven days after IP injection of 10^9 sheep erythrocytes, blood was collected by cardiac puncture from chloroform-anesthetized animals into 3.2% sodium citrate. After centrifugation, the plasma was heat inactivated, and serial (1:1) dilutions were made in phosphate buffered saline. To each of the dilutions in a microtiter dish, an equal volume of 0.5% suspension of sheep erythrocytes was added. After incubation for 2 hr at 37°C, the plates were observed on a magnifying mirror for agglutination of the erythrocytes. The antibody titers were expressed as $\log_2$ of the reciprocal of the first dilution where there was no visible agglutination.

Cell-Mediated Immunity

Cell-mediated immunity was evaluated by measuring the delayed hypersensitivity (DTH) response to sheep erythrocytes. This is a modification of the methods of Legrange et al. and Paranjpe et al. (*4*, *5*). Sensitization was accomplished by injecting 10^8 sRBC in a volume of 20 μl into the left footpad (LFP). Four days following sensitization, the mice were challenged in the LFP with 4 × 10^8 sRBC in a volume of 40 μl. At 17 hr following challenge, the mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (HSA) (~80,000 cpm/0.1 ml). Two hours later, the mice were sacrificed by cervical dislocation and both hind feet were removed at the ankle joint and radioassayed in a gamma counter. The RFP served as an unchallenged control for background ^{125}I-HSA. A group of mice which were not sensitized but were challenged as above acted as unsensitized controls to determine nonspecific swelling. Results are expressed as a stimulation index (SI), which is calculated as follows:

$$\mathrm{SI} = \left[\frac{\text{LFP sensitized}}{\text{RFP sensitized}}\right] - \left[\bar{X}\left(\frac{\text{LFP unsensitized}}{\text{RFP unsensitized}}\right)\right]$$

Spleen Cell Response to Mitogens

Lymphocyte responsiveness was assessed using the B-cell mitogen LPS, which is lipopolysaccharide from *Salmonella typhosa* 0901 (Difco), and the T-cell mitogen concanavalin A. For mitogenicity assays, spleens were removed aseptically and pushed through a stainless steel 80-mesh screen to provide a single cell suspension. Cells were diluted to 5 × 10^6/ml in RPMI 1640 medium supplemented with 2 *mM* L-glutamine and 10% heat-inactivated fetal calf serum. A 100 μl portion of the cell suspension was added to each of three replicate wells into previously prepared microtiter plates containing 50 μl of the mitogen, which had been stored at -70°C. LPS was used in amounts of 1, 5 and 20 μg per well; concanavalin A was used in amounts of 1, 5 and 10 μg per well. Plates were incubated at 37°C in 10% CO_2 and 95% humidity for 48 hr. The cells were then pulsed with an I-iododeoxyuridine/fluorodeoxyuridine solution (0.2 μCi IUdR in 1μ*M* FUdR). After an 18-hr incubation, the cells were collected on filter discs using a Titertek cell harvester and counted in a Beckman gamma counter.

Functional Ability of the Reticuloendothelial System

We have previously compared several particles for measuring the phagocytic activity of the fixed macrophage system (*6*). For these studies we used sRBC.

Freshly drawn sRBC (5×10^9 cells/ml) were radiolabeled with ^{51}Na chromate (NEN) in a 37°C shaker bath with 1 mCi/5 ml of cells for 30 min. After chromation, the sRBC were washed with Alsever's solution until the supernatant was virtually radioactive free. Unlabeled sRBC (5×10^9 ml) were added to the labeled cells until the hematocrit was approximately 10% The resulting CPM's were approximately 200,000/0.1 ml. The sRBC were refrigerated and used the following day. Before use, the cells were washed to remove any free chromium released overnight.

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 29°C. At zero time, 0.1 ml of labeled particle/10 g body weight was injected intravenously. Blood samples (10 μl) were taken from the tip of the tail at 2, 4, 6, 8, 10 and 15 min. The blood samples were put into 1 ml of distilled water and radioassayed. At the end of 60 min, the mice were sacrificed by decapitation and drained of blood. The liver, spleen, lungs, thymus and kidneys were removed, weighed and placed in tubes for counting in a gamma counter. Blood clearance was expressed as the phagocytic index, which is determined by the slope of the clearance curve. Organ distribution was expressed as percent organ uptake and cpm/mg tissue (specific activity).

Statistical Evaluation

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's *T*-test was performed (*7*). Values which differ from vehicle control at $p < 0.05$ are noted in tables. Each of the values is given as the mean ± standard error (S.E.) of the mean, except the historical controls, which are shown with both standard deviation and standard error.

Results

Acute Toxicity

The acute toxicity of each of the chemicals was determined to provide range-finding doses to be used in the subchronic studies and possibly implicate target organs for toxicity.

The LD_{50} of dichloroethane administered as a single gavage was 489 (424-552) and 413 (337-4[illegible]9) mg/kg for male and female CD-1 mice, respectively. (The numbers in parentheses represent the 95% confidence limits.) The mice died over a 48-hr period. Those surviving 48 hr recovered and appeared normal at the end of the 14-day observation period. Upon gross inspection, the target organs for the acute effects appeared to be the lungs and liver.

In contrast to dichloroethane, the LD_{50} for dichloroethylene was 2221 (1987-2469) and 2391 (2056-2788) mg/kg in male and female CD-1 mice, respectively. The mice died between 2 and 24 hr, apparently by central nervous system suppression. The LD_{50} of trichloroethylene was 2402 (2065–2771) mg/kg for male mice and 2454 (1040–3062) mg/kg for female mice, with the mice dying over a 9-day period. At the high doses, the deaths occurred within 24 hr, most probably of central nervous system suppression; the lower lethal doses caused deaths later, presumably with the liver and kidney being the target organs.

Dexamethasone was administered at doses up to 1000 mg/kg without consistent lethal effects. Doses higher than this were not used because subchronic toxic doses were known to be less than 10 mg/kg.

Subchronic 14-Day Studies

The subchronic 14-day exposures served to provide range-finding doses that were used to establish the doses for subchronic 90-day studies. These data from the 14-day exposures can also be used to supplement other information in establishing acceptable exposure levels in acute environmental spills.

CD-1 male mice were administered the organic water contaminants dichloroethane, dichloroethylene and trichloroethylene daily for 14 days by gavage at doses 0.01 and 0.1 times the LD_{50}. This exposure did not alter body weight over the 14-day

Table 1. Hematological and coagulation values of CD-1 mice gavaged for 14 days with three organic water contaminants.[a]

Treatment	Parameter	1,2-Dichloroethane	1,2-Dichloroethylene	1,1,2-Trichloroethylene
Control	Leukocytes × 10^{3}/mm^{3}	8.24 ± 0.94	10.06 ± 1.02	5.93 ± 0.46
	Hematocrit, %	39 ± 0.9	42 ± 0.7	42 ± 0.5
	Hemoglobin, g-%	13 ± 0.4	14.4 ± 0.4	12.2 ± 0.3
	Fibrinogen, mg/dl	280 ± 8.0	371 ± 11.0	283 ± 5.0
	Prothrombin time, sec	9.1 ± 0.2	6.9 ± 0.1	8.5 ± 0.1
0.01 × LD_{50}	Leukocytes × 10^{-3}/mm^{3}	7.60 ± 0.52	8.66 ± 1.17	5.15 ± 0.44
	Hematocrit, %	40 ± 0.6	42 ± 0.7	42 ± 0.4
	Hemoglobin, g-%	12.8 ± 0.3	14.4 ± 0.4	12.7 ± 0.3
	Fibrinogen, mg/dl	262 ± 6.0	373 ± 15	294 ± 7
	Prothrombin time, sec	9.1 ± 0.1	6.8 ± 0.1	8.5 ± 0.1
0.1 × LD_{50}	Leukocytes × 10^{-3}/mm^{3}	5.76 ± 0.49[b]	7.44 ± 0.53	6.15 ± 0.38
	Hematocrit, %	41 ± 0.5	42 ± 0.1	39 ± 0.7[b]
	Hemoglobin, g-%	12.9 ± 0.1	14.2 ± 0.2	11.4 ± 0.5
	Fibrinogen, mg/dl	278 ± 8	327 ± 13	272 ± 8
	Prothrombin time, sec	9.0 ± 0.1	6.4 ± 0.1	8.5 ± 0.1

[a] CD-1 male mice were exposed to daily gavage of the organic water contaminant for 14 days. Twenty-four hours after the last gavage, blood was collected by cardiac puncture into 3.2% sodium citrate. The values represent the mean ± S.E. derived from 10-12 mice per group.

[b] $p < 0.05$ compared to control.

period. The average initial weights were approximately 30 g. During a necropsy performed 24 hr after the last chemical administration, the liver, spleen, lungs, thymus, kidney, and brain were weighed. There were no changes seen in the organ weights for dichloroethane and dichloroethylene. Trichloroethylene produced a 33% increase in liver weight at the high dose.

Blood obtained by cardiac puncture was analyzed for selected hematological and coagulation parameters (Table 1). Dichloroethane caused a 30% decrease in leukocyte number. Trichloroethylene produced

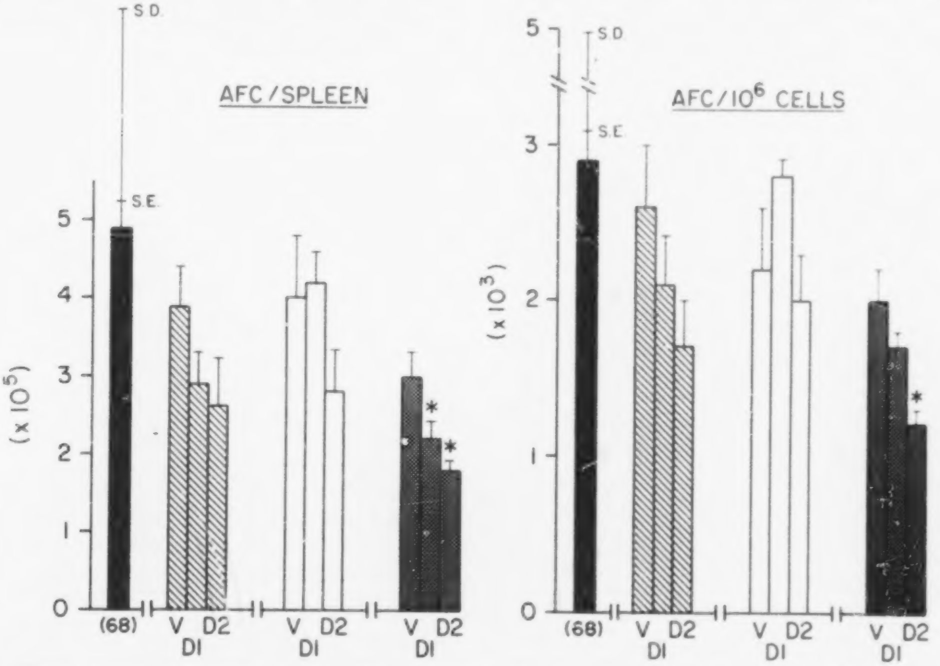

FIGURE 1. Humoral immune response to sheep erythrocytes. CD-1 male mice were exposed by gavage to (■) dichloroethane, (□) dichlorethylene and (▩) trichlorethylene at 0.01 (D1) and 0.1 (D2) × LD_{50} dose, daily for 14 days. Antigenic sensitization was performed on day 11 and antibody response measured 24 hr after the last gavage. The bar represents the mean ± S.E. derived from 10-12 mice per group, except the historical controls (■), which show the mean ± S.D. ± S.E. derived from 68 mice. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

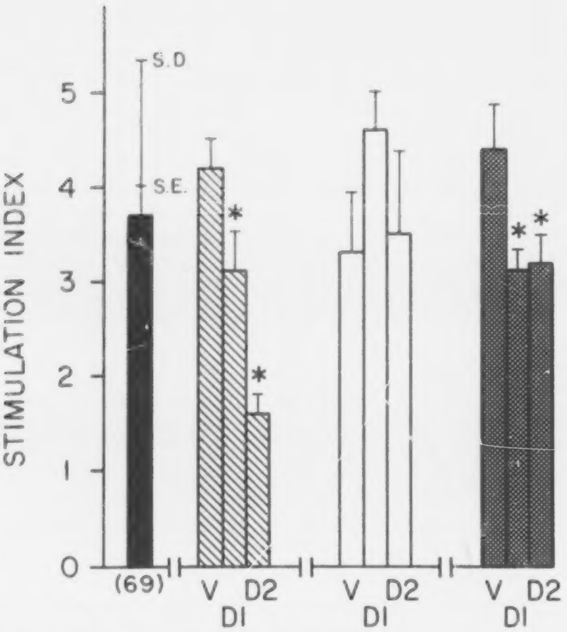

FIGURE 2. Cell-mediated immune response to sheep erythrocytes. CD-1 male mice were exposed by gavage to (■) dichloroethane, (□) dichloroethylene and (▩) trichloroethylene at 0.01 (D1) and 0.1 (D2) × LD_{50} dose, daily for 14 days. Antigenic sensitization was performed on day 11, challenge was carried out on day 15, and delayed hypersensitivity quantified 19 hours after challenge. The bar graph represents the mean ± S.E. derived from 12 experimental controls (V) and 10 chemically exposed mice. The historical controls (■) are the mean ± S.E. ± S.D. derived from 69 mice. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

a 7% ($p < 0.05$) decrease in hematocrit and a concomitant decrease ($p < 0.1$) in hemoglobin.

Serum obtained by cardiac puncture was analyzed for lactic dehydrogenase, serum glutamic pyruvate transaminase, and blood urea nitrogen levels. There were no elevations in any of these parameters after 14 days exposure to the three organic compounds (data not shown).

The humoral immune response was assessed by measuring the number of IgM antibody forming cells (AFC) to sRBC. On the AFC/spleen basis, all three chemicals showed a trend towards producing immunosuppression (Fig. 1). However, the only chemical which showed an effect at the $p < 0.05$ level was dichloroethane, which produced a 25 and 40% suppression at 4.9 and 49 mg/kg, respectively. The suppression of AFC/spleen produced by trichloroethylene and dichloroethylene was significant at $p < 0.1$.

Cell-mediated immune response was measured by quantifying the extravasation of human ser um albumin during the delayed hypersensitivity respo se to sRBC (Fig. 2). Trichloroethylene produced 25 and 60% suppression in the DTH response at 24 and 240 mg/kg, respectively. Dichloroethylene sho ed no effect, while dichloroethane produced a sli ht effect which was not dose-dependent.

Subchronic 90-Day Study

Based upon data derived from the subchr ic 14-day study, the concentrations of the chemi ls in the drinking water solutions were set so a to deliver daily doses equivalent to those administe ed by gavage. The calculations were based on ur data showing that male and female mice dr nk about 9 or 7 ml/day, respectively. For dichloroetha e, the desired daily doses were 4.9, 49 and 490 mg/ g. The 490 mg/kg dose was added because the d ta from the subchronic 14-day exposure showed t at doses higher than 49 mg/kg could be tolerated.

The concentrations for dichloroethane in the drinking water were 0.02, 0.20 and 2.0 mg/ml. The calculated time weighted average doses delivered based on actual fluid consumed were 3, 24 and 189 mg/kg. The differences between the expected doses and actual doses delivered were caused by decreased fluid consumption in the middle and high dose groups.

In the case of dexamethasone, the concentrations in the drinking water were set at 0.1, 1.0 and 2.0 μg/ml, with the expected doses being 0.024, 0.24 and 0.49 mg/kg. The doses delivered based on fluid consumption were 0.02, 0.2 and 0.4 mg/kg.

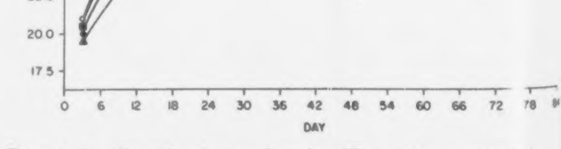

FIGURE 3. Growth chart of male CD-1 mice expose to 1,2-dichloroethane in the drinking water: (■) deion ed water; (●) 3 mg/kg delivered, 0.1 mg/ml in water; (○ 24 mg/kg delivered, 1.0 mg/ml in water; (Δ) 189 mg/kg d ivered, 2.0 mg/ml in water. There were 48 mice in the deionized water group, and 32 mice in each of the dexamethasone-exposed groups.

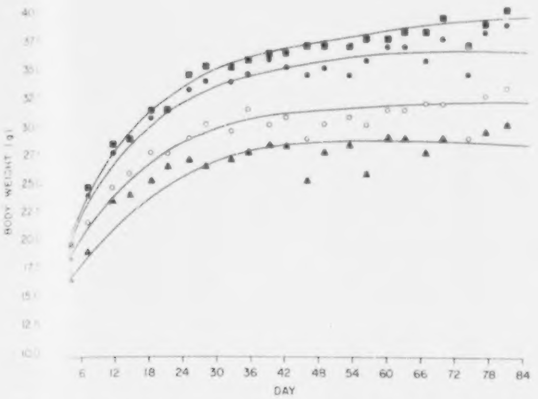

FIGURE 4. Growth chart of male CD-1 mice exposed to dexamethasone in the drinking water: (■) deionized water; (●) 0.04 mg/kg delivered, 0.1 μg/ml in drinking water; (○) 0.2 mg/kg delivered, 1.0 μg/ml in drinking water; (▲) 0.4 mg/kg delivered, 2.0 μg/ml in drinking water. There were 48 mice in the deionized water group and 32 mice in each of the dexamethasone-exposed groups.

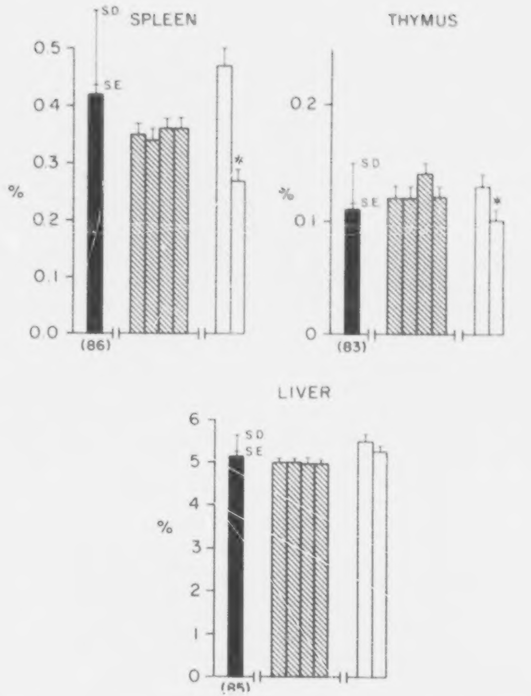

FIGURE 5. Organ weights (as % of body weight) of CD-1 mice exposed to dichloroethane or dexamethasone: (■) historical controls (numbers in parentheses represent number of mice); (▨) dichloroethane at 0, 3, 24, and 189 mg/kg; (☐) dexamethasone at 0 and 0.4 mg/kg. The bars represent the mean ± S.E., except for the historical control, where the S.D. is also presented. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental control.

The growth curves for mice exposed to dichloroethane and dexamethasone in the drinking water are shown in Figures 3 and 4. Both compounds caused a concentration-dependent decrease in growth rate. However, fluid consumption was unaltered by the presence of dexamethasone, as seen in 5.0, 4.5, 4.5 and 4.5 ml/mouse/day consumed for the 0, 0.01, 1.0 and 2.0 μg/ml dose groups, respectively. Fluid consumption in mice exposed to dichloroethane was decreased in relationship to the concentration, as seen in 5.0, 5.5, 4.2 and 2.8 ml/mouse/day consumed for the 0, 0.02, 0.2 and 2.0 mg/ml dose groups, respectively. As will be seen, decreased growth rate alone, or decreased fluid consumption alone does not predispose the immune system to altered reactivity.

Organ Weights. Dichloroethane did not alter the weights of the liver, spleen, lungs, thymus or kidneys at the levels of exposure. The weights, expressed as percent of body weight for the liver, spleen and thymus, are shown in Figure 5. The absence of effects on organ weights was also seen when the data were expressed on an organ to brain ratio or in milligrams of tissue. The positive control, dexamethasone, administered at a dose of 0.4 mg/kg, showed a 42% decrease in spleen weight and a 30% decrease in thymus weight, with no changes detected in liver weight (Fig. 5).

Hematology. Hemoglobin, hematocrit, erythrocytes, leukocytes, and platelets were not altered by exposure of mice to dichloroethane. The leukocyte data are shown in Figure 6. In contrast, mice receiving dexamethasone showed a 24% decrease in total leukocytes and 43% decrease in the percent of peripheral blood lymphocytes. This lymphopenia was accompanied by a corresponding increase in polymorphonuclear leukocytes. There were no exposure-related changes in the number of blood monocytes.

Humoral Immunity. The number of IgM spleen antibody forming cells (AFC) was quantitated on days 4 and 5 after sRBC immunization. In separate groups of mice, serum hemagglutination antibody levels were determined seven days after sRBC immunization. The AFC responses of mice exposed to dichloroethane and dexamethasone are shown for the peak day of response, which was day 4 (Fig. 7). Dichloroethane exposure caused no significant change in the number of AFC/spleen or per 10^6 nucleated spleen cells. A 55% reduction in AFC/spleen was seen in dexamethasone exposed mice. However, the specific activity, i.e., AFC/10^6

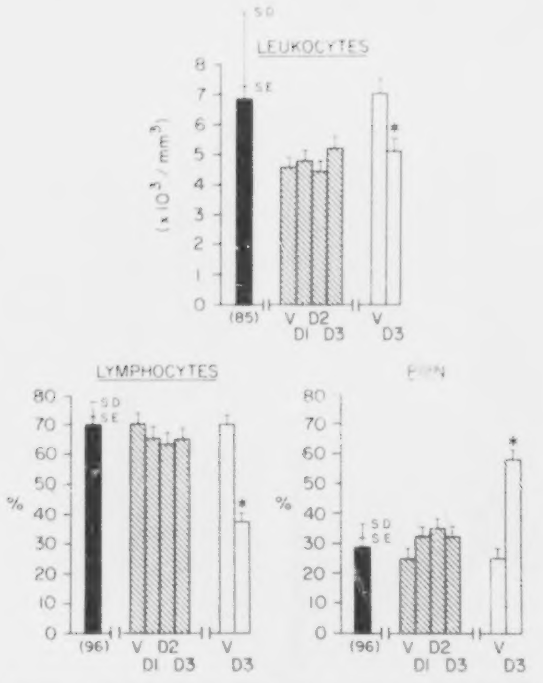

FIGURE 6. Leukocyte and differential counts of mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = 0 mg/kg, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = 0 mg/kg and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. PMN = polymorphonuclear leukocytes. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental controls.

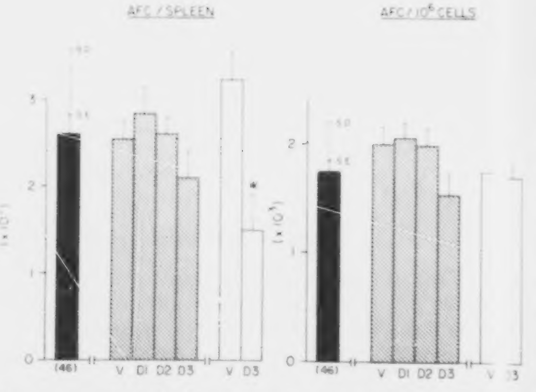

FIGURE 7. Humoral immune response to sRBC of CD-1 mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = 0 mg/kg, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = 0 and D1 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 mice in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked by an asterisk (*) indicate $p < 0.05$ compared to experimental control group.

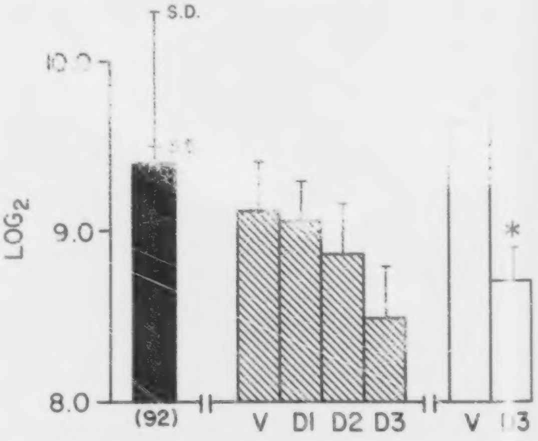

FIGURE 8. Serum hemagglutination titers in CD-1 male mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = deionized water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = deionized water, D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 mice in each exposure group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to experimental control group.

spleen cells, was not altered, indicating that the reduction in spleen size (Fig. 5) and spleen cell number (data not shown) was not specific for antibody-producing cells. The serum antibody titer in the dexamethasone-exposed mice was reduced from 1/787 to 1/415, a 48% suppression, confirming the results seen in AFC response (Fig. 8).

There appeared to be a dichloroethane dose-dependent reduction in hemagglutination titer. Although this was not significant at the $p < 0.05$ level, it is important to note that AFC/spleen and AFC/10^6 spleen cells were also reduced, albeit not significant at the $p < 0.05$ level.

A more complete presentation of the dexamethasone suppression of humoral immunity is seen in Figure 9. The peak day of response was day 4. Dose-dependent suppression was seen on both days 4 and 5 on an AFC/spleen basis. On day 4, there was no effect when presented as AFC/10^6 spleen cells, but there was a slight suppression on day 5.

Bacterial lipopolysaccharide (LPS) is a known B-cell mitogen. The spleen cells of dichloroethane

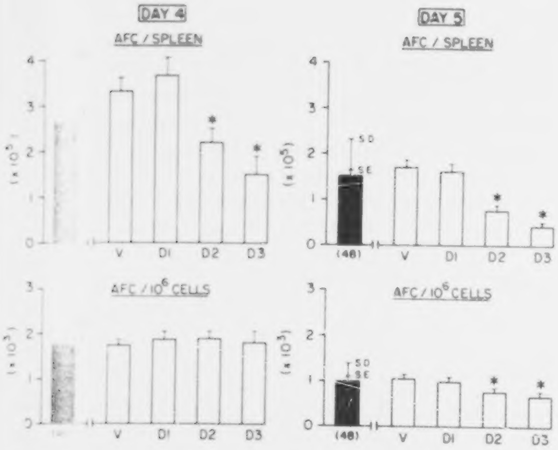

FIGURE 9. Humoral immune response to sRBC in CD-1 mice exposed to dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (□) dexamethasone at V = distilled water, D1 = 0.02 mg/kg, D2 = 0.2 mg/kg, and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in the deionized water group and 16 in each dexamethasone-exposed group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to distilled water (control) group.

and dexamethasone were evaluated for their ability to respond to this mitogen. The data seen in Figure 10 show that neither dichloroethane or dexamethasone alters the spleen cell response to three concentrations of LPS. The spleen cell response of control mice in the dexamethasone experiment was at the upper limits of the historical controls and was about 1.8 times that of the control mice in the dichloroethane experiment.

Cell-Mediated Immunity. The DTH response to sRBC was used as an *in vivo* indicator of the status of cell-mediated immunity. Mice exposed to dichloroethane showed no significant change in the DTH response. Mice exposed to dexamethasone showed a 65% reduction in the DTH response. Flumethasone injected parenterally also produced a marked suppression of 64% (Fig. 11). These data do not provide insight into which arm of the DTH response (afferent or efferent) was the site of action.

Spleen cell responsiveness to concanavalin A, a specific T-lymphocyte mitogen, was evaluated in mice exposed to dichloroethane and dexamethasone. The results are shown in Figure 12. Three concentrations of mitogen were used, producing the expected bell-shaped curve. This is seen by examin-

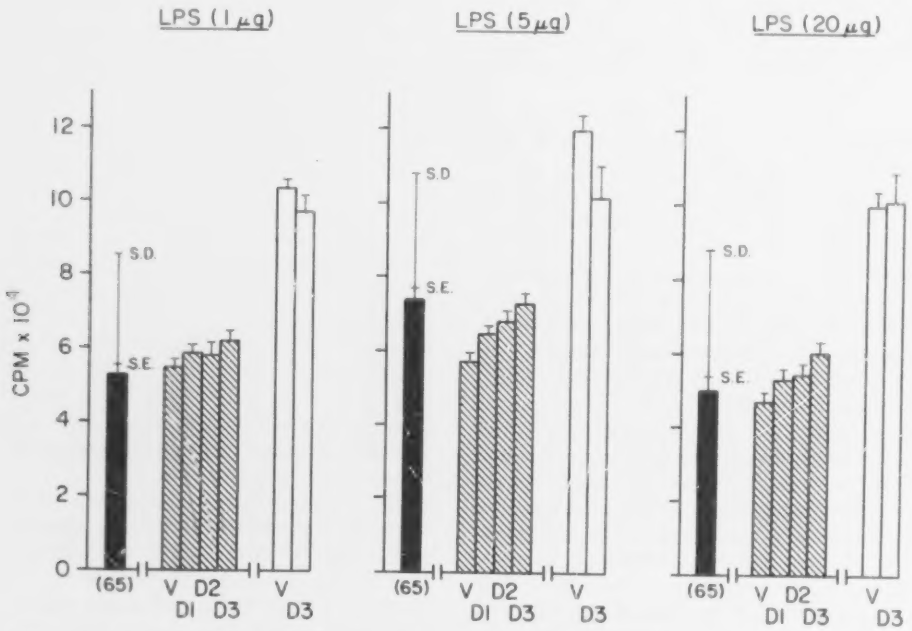

FIGURE 10. Spleen cell responsiveness to lipopolysaccharide (*S. typhose* 0901): (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (□) dexamethasone at V = distilled water, and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in the deionized water group and 16 in each of the dichloroethane- and dexamethasone-exposed groups.

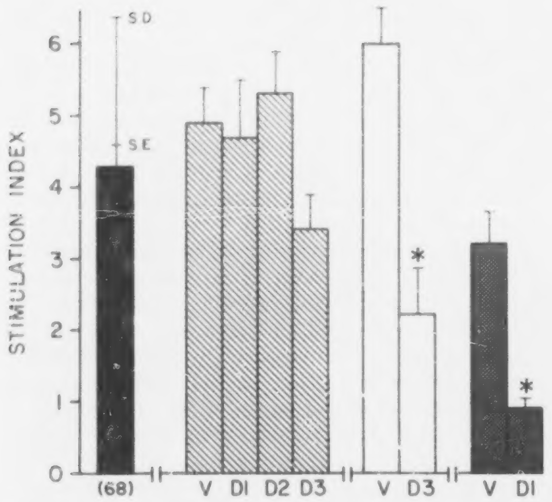

FIGURE 11. Cell-mediated immune response to sheep erythrocytes in CD-1 male mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = distilled water, D3 = 0.4 mg/kg; (■) flumethasone at V = 0.15*M* sodium chloride IP daily for 3 days, D1 = 5 mg/kg IP daily for 3 days. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in each of the experimental control groups and 16 mice in each chemically exposed group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control group.

ing the historical and experimental controls. Dichloroethane exposure caused no alteration in spleen cell response to concanavalin A, except for the low dose group at the low (1 μg) concentration of mitogen. The results for dexamethasone were different than expected: at every concentration of mitogen, the response was significantly enhanced. In contrast, *in vitro* exposure of dexamethasone always produced a suppression of lymphocyte response to the mitogen, with an ED_{50} of about 5 μg/5 × 10^6 spleen cells (data not shown).

Functional Activity of the Reticuloendothelial System. There are many assays for macrophage function. For these studies, the vascular clearance rate and organ distribution of ^{51}Cr sRBC were used. Mice exposed to dichloroethane and dexamethasone showed no significant change in the rate of clearance, which is primarily a function of liver and spleen phagocytosis (Fig. 13). Flumethasone, administered parenterally for three days, caused a 67% reduction in clearance rate.

Liver, spleen, lung, thymus, and kidney uptake of ^{51}Cr sRBC were determined 1 hr after IV administration. This was the time previously determined to be the plateau phase of uptake. The results for the spleen, thymus, and liver of mice exposed to dichloroethane and dexamethasone are shown in Figure 14. Dichloroethane produced no change in phagocytic uptake in the organs analyzed. Mice exposed to dexamethasone showed a reduced uptake in the spleen and thymus, but not the liver. The size of the spleen and thymus was reduced by this exposure (Fig. 5). Calculated as specific activity, i.e., cpm/mg, there was no suppression of phagocytosis in the organs examined.

Discussion

Successful interfacing of immunological assays into the toxicological evaluation of chemicals is in its early stages. We present here an approach to immunotoxicity assessment from the viewpoint of the toxicologist. After acute toxicity studies, which provide a basis for dose levels and potential target organs, subchronic 14- and 90-day studies are conducted. Immunotoxicity studies are not conducted after acute administration because of the difficulties associated with the time of antigen presentation in relationship to the time of chemical administration. In the subchronic studies, the antigens are given during the exposure period, which allows for the target cells to be at some equilibrium with the chemical during the pre- and post-antigen period. The route relevant to human exposure is used in random-bred male and female mice and the sensitivity of the immune system is evaluated against a background of the more standard toxicological methods.

Of the ten chemicals studied over the last five years, we selected dichloroethane, dichloroethylene and trichloroethylene to represent subchronic 14-day exposure, and dichloroethane and dexamethasone for subchronic 90-day exposure. Dichloroethane is used to follow a single agent through the immunotoxicological evaluation and also to show that reduction in body weight gain alone does not *a priori* predispose the immune system to toxicity. Dexamethasone is used as a positive control for many of the immunological assays.

The historical control data are presented along with the experimental control data to provide a picture of the variability in the assays. It is important to reiterate that these assays are performed in random-bred mice and the relationship of immune responses to the immune response genes are well known. Refinement of immunotoxicological assays benefits by maintaining and referring to historical controls.

Dichloroethane at concentrations as low as 1/100

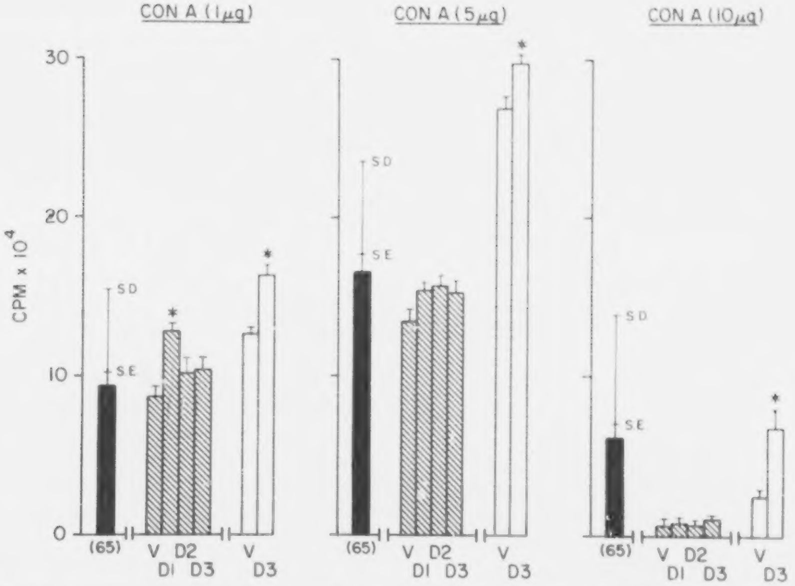

FIGURE 12. Spleen cell responsiveness to concanavalin A: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = distilled water and D3 = 0.4 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for the historical controls. There were 24 mice in each of the distilled water groups and 16 in each of the dichloroethane- and dexamethasone-exposed groups. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

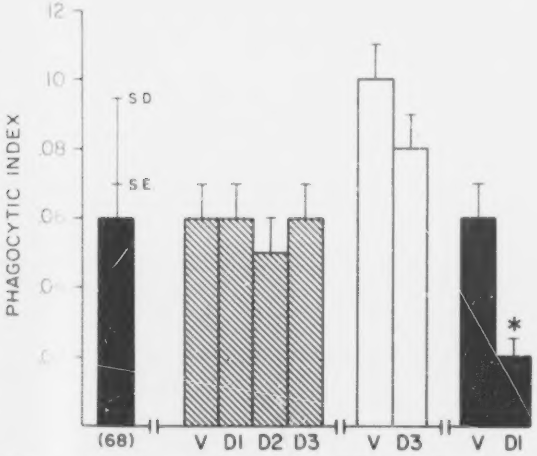

FIGURE [illegible]. Vascular clearance rate of sRBC in CD-1 mice exposed to dichloroethane, dexamethasone and flumethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = [illegible] mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = distilled water, D3 = 0.4 mg/kg; (■) flumethasone at V = distilled water, D1 = 0.5 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in each of the distilled water groups and 16 mice in each exposure group. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

of the LD_{50} produced a suppression of both humoral and cell-mediated immunity when administered daily for 14 days by stomach tube. In the subchronic 90-day study, where doses as high as 189 mg/kg (0.4 of the LD_{50}) were consumed in the drinking water, there were no significant ($p < 0.05$) effects, although there were trends toward suppression at the high exposure level. There are at least two explanations for these results. First, the level at the biophase, i.e., immunocompetent cell, may be higher with the bolus presentation than with semicontinuous self-administration in the drinking water. Second, dichloroethane may induce its own metabolism over the longer exposure period, effectively reducing the amount of chemical reaching the immune cells.

Dexamethasone provides some interesting effects on the immune system. In these studies, it was administered in the drinking water, caused a dose-dependent decrease in body weight gain (with no change in fluid consumption), and unexpectedly did not markedly alter other toxicologic parameters such as hematology or clinical chemistries. In the case of humoral immunity, dexamethasone produced a dose-dependent reduction in AFC/spleen, but not an equivalent suppression of AFC/10^6 spleen cells, suggesting a nonspecific cytotoxic effect on the splenocytes. This is supported by the reduction in

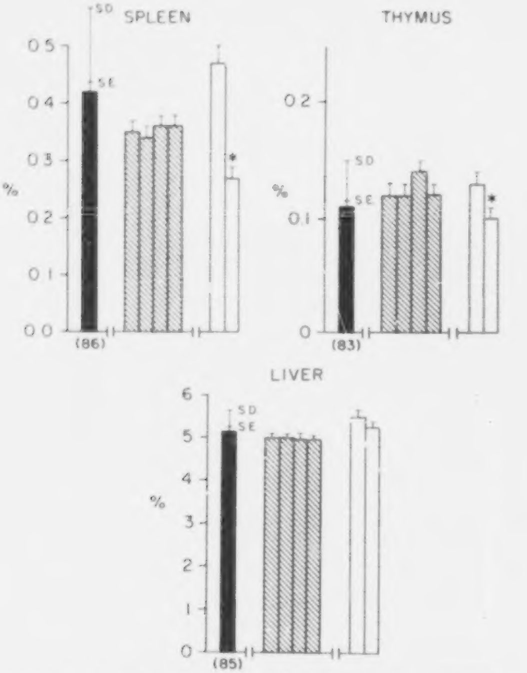

FIGURE 14. Organ uptake of sRBC in CD-1 mice exposed to dichloroethane and dexamethasone: (■) historical controls (numbers in parentheses represent the number of mice); (▨) dichloroethane at V = distilled water, D1 = 3 mg/kg, D2 = 24 mg/kg, and D3 = 189 mg/kg; (☐) dexamethasone at V = distilled water and D3 = 0.3 mg/kg. The bars represent the mean ± S.E. The S.D. is also shown for historical controls. There were 24 mice in each of the distilled water groups and 16 mice in each of the exposure groups. Bars marked with an asterisk (*) indicate $p < 0.05$ compared to control groups.

serum antibody levels to sRBC and a normal B-lymphocyte mitogen response to LPS. In the mitogen assay, a constant number of spleen cells were added to the microtiter well, thus a reduction only in cell number would not alter mitogen response.

The effects on cell-mediated immunity present a more complex picture. There was a marked dose-dependent reduction in DTH response to sRBC in the presence of a hyper-responsiveness to the T-lymphocyte mitogen, concanavalin A. The increased response to concanavalin A could be caused by a change in T- and B-lymphocyte pool size. That is, a slightly greater cytotoxicity to the B-lymphocyte could result in more T-lymphocytes available in the spleen, thus more T-lymphocytes available to respond to the mitogen. This does not explain the suppression of the DTH response. Since steroids are excellent inhibitors of the efferent arm of the immune response (anti-inflammatory activity), it is quite likely that the reduced DTH response with this type of exposure is caused only by the effect on the expression of the DTH, but not on the development of the sensitized lymphocytes. This is supported by the data showing that the popliteal lymph node proliferation in these animals was not significantly affected (data not shown).

Approaches and results from these types of studies provide a good basis for evaluating a chemical's potential risk to the immune system.

This work was supported in part by a grant from the Environmental Protection Agency (R806481010) and a contract from the U. S. Army Medical Research and Development Command (DAMD 17-78-C-8083).

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Environmental Health Perspectives
Vol. 43, pp. 53-59, 1982

Immunotoxicology in the Pharmaceutical Industry

by Kenneth C. Norbury*

Development of an immunotoxicology program within the pharmaceutical industry is described. With few guidelines in this area and a multitude of factors to consider, a basic screen for evaluating immune competence in species routinely used in toxicologic studies has been proposed. The future of immunotoxicology depends upon the ability of the selected immune function tests to be predictive of human risk.

The use of immunologic methods over the past several decades has been important in the development of anti-inflammatory drugs. However, the application of immunology within a toxicology environment has only occurred relatively recently. The Immunotoxicology Program at Merck Sharp & Dohme Research Laboratories (MSDRL) has originated within the Department of Safety Assessment. I would like to review the development of this program since its inception three years ago. Since our program is still in its infancy, I must emphasize this paper represents many of my opinions based on the state-of-the-art and future application of immunotoxicology as I perceive them. Because there remains a considerable amount of characterization and refinement of our immune function tests, we do not intend to test new drug candidates until the utility of the procedure is proven.

Historical Perspective

In 1977 there were few guidelines regarding immunotoxicologic testing. The European Economic Community (EEC) drafted the following purpose of immune function tests in the fall of that year: (1) to evaluate the potential risk of the drug by determining the functional significance of the effect on lymphoid organs found in routine toxicity studies; (2) to obtain more insight into the mode of action (i.e., which cell type is the target and which phase of the immune response is impaired).

At first, this may seem to be a reasonable attempt to acknowledge the importance of immunologic testing, but I call to your attention one particular paragraph in these proposed guidelines:

> At the termination of a toxicity study, thymus, spleen, and lymph nodes should be weighed and examined microscopically. From these data and measurements of serum immunoglobulin fractions and counts of circulating lymphocytes, the conclusion should be reached whether the substance has an effect on the lymphoid system and whether or not specific function tests have to be performed.

The intent, then, is for immune function tests to be done only after histopathologic and hematologic studies have been performed. There are some important pitfalls to this approach. The first is that histopathologic changes are not a sensitive indicator of drug-induced immunologic toxicity. Generally, histomorphologic changes associated with compromised immune function are seen only at fairly high dosage levels. In addition, the inability to differentiate morphologically between T-cells and B-cells is well known. Serum immunoglobulin fractions are equally insensitive as indicators of immune function. They represent an immune response that occurred early in life and are not representative of immune competence in older individuals. The circulating leukocyte count and differential, while

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admittedly important in determining immune competence, could be misleading if absolute numbers are within the normal range. The function of these cells might still be impaired.

None of these tests, with the possible exception of serum immunoglobulin levels, really measures immune function. Yet the EEC guidelines did recommend that certain immune function tests be done, patterned after the review by Vos (*1*). The scope of these assays involves *in vivo* and *in vitro* testing of cell-mediated immunity (CMI), humoral immunity (HI) and phagocytosis by macrophages. As we shall see later, this approach needs to be tempered to meet the needs of conventional toxicity studies.

Factors That Influence Immunotoxicological Testing

A standard operating procedure in toxicology includes total leukocyte counts and differential, measurement of organ weights (such as adrenal and spleen), and histologic examination of lymphoid organs (spleen, thymus, lymph nodes, and bone marrow). Certain immune function tests would then have to be added to the conventional study in order to make a valid interpretation of immunotoxicologic effects. The panel discussion at the Gordon Conference on Toxicology and Safety Evaluations in August of 1978 did a great deal to prioritize the kinds of determinations that need to be done and which function tests are appropriate to do. On the other hand, some laboratories have proposed a more extensive and perhaps idealistic approach. MSDRL has elected to pursue an approach which applies the most current knowledge of immunology in a practical way.

There are many considerations which tend to complicate an analysis of immune competence. Dosage level and frequency are important variables to consider. Low dosage levels may enhance an immune response whereas higher dosage levels may be immunosuppressive. Whether a drug is administered as a single or multiple dose, as well as the route of administration, will contribute to the effect observed. Furthermore, it is important to correlate immune function tests with the dosage regimens used. All too frequently, substantially different protocols are used to measure CMI and HI, with particular reference to the time of administration of the test compound with respect to the time the immunologic stimulus (antigen) is introduced. Consequently, any differences between immune responses are not easy to interpret. Finally, the age of the animals being tested is particularly critical from an immunologic standpoint. The prenatal period, early postnatal period and late adulthood may be especially vulnerable to immunologic alterations. In fact, there does appear to be a natural loss of immunoreactivity with age (*2*).

Another factor would be the species employed for immunologic testing, taking into consideration drug metabolism. For example, the difference in the LD_{50} between mice and rats for cyclophosphamide (*3*) most likely represents differences in metabolism to the reactive intermediate. One fundamental difference between routine toxicologic evaluation and basic research in immunology is the use of random-bred versus inbred strains, respectively. Random-bred strains may accentuate animal-to-animal variation, especially since many immune responses are under genetic control (*4*). The adaptation of universally accepted assays in one species (i.e., mouse) to a species typically used in toxicology (e.g., rat) requires adequate characterization. For instance, one should not assume that a B-cell mitogen (lipopolysaccharide) in the mouse is automatically a B-cell mitogen in the rat.

Secondary factors resulting from stress-related phenomena (i.e., adrenocortical hypertrophy) can also lead to immunologic changes. Other important considerations include the relative sensitivity of immunologic assays, method of calculation, correlation of *in vivo* and *in vitro* findings, differences between sexes, production of delayed or biphasic immunobiologic responses, presence of contaminants in the feed or drinking water and the length of the study.

The ultimate proof of an immunologic evaluation is the ability to predict human risk. One of our most frequently asked questions is: what does it mean if the hematologic, serum biochemical and gross and histopathologic examinations show no effect due to treatment, but an immune function test indicates an alteration? The answer, of course, depends upon the predictability of the immune function test. A more pragmatic approach might be to ask whether a given compound will cause hypogammaglobulinemia, alter the state of hypersensitivity to pollens, food or contact allergens, increase susceptibility to infection, alter complement activity or induce an autoimmune reaction, all of which are highly relevant questions regarding human risk. Indeed, determination of serum immunoglobulin levels, induction of delayed type hypersensitivity reactions in skin, quantitation of specific antibody titers, determination of lymphoproliferative potential, and enumeration of T and B-cells form the basis of clinical immunology.

It is important to recognize that the evaluation of im une competence is related to immune responsive s and is not simply the examination of lymp id tissue at a single point in time using hist thologic techniques and hematology. Four step f the immune response that may be altered by c gs are: (a) the recognition of antigen, (b) clon roliferation, (c) differentiation and (d) the effe r phase, characterized by the infiltration of imm e cells into the site of an immunologic reac n and the secretion of lymphokines, induction delayed hypersensitivity, graft rejection, and/ elaboration of specific antibodies. Drugs that lock contractile microfilaments, such as cytoc lasin B, might be expected to interfere with e antigen processing by macrophages. The majo y of antineoplastic drugs are known to inter upt the proliferative phase of lymphocytes by bl king DNA synthesis.

In summary, the scope of any immunotoxicology program should be to develop tests that can reliably, efficiently, and inexpensively screen a large number of compounds for their ability to modify the immune response. Because of the complexity of the immune system, a panel of tests, both *in vitro* and *in vivo* must be used. Testing should proceed in basically two phases: simple, straightforward assays that are capable of detecting major alterations in the immune response (such as antibody production to a T-dependent antigen which requires helper T-cells, macrophages, and B-cells) followed by more complicated procedures (specific immune models) that elucidate mechanisms of drug actions and identify cell types.

Development of an Immunotoxicology Program

At MSDRL we started with the assumption that the e luation of immune competence would be perfo ed during a routine toxicity study. This appr h offers several advantages, including the coor tion of immunologic tests with other toxicolog l parameters (i.e., hemotology, serum biochem y, and histopathology), collection of multiple od samples, potential to build a large histo l data base and detection of novel immune mod ors that would not have otherwise gone thro an immunologic screen. However, before imm toxicology can begin to pay dividends, prog s will need to be made in some areas, such as in asing our understanding of the mechanisms invo d in the immune response, adapting immunolo methods from one species to another to obta interspecies correlation of data, arriving at a co ensus on which assays should be performed and accepting a multifaceted approach to delineate the mechanism of drug action (not necessarily in that order).

Originally, three fundamental immunologic function areas were designated. The primary objectives were to select tests that could be done in rats, dogs, and monkeys and would interface with the standard operating procedures of our laboratory, such as the collection of blood samples at the same time as routine bleedings for hematologic and serum biochemical studies.

Serum Immunoglobulin Levels

The quantitation of serum immunoglobulin levels, specifically IgG, IgM, and IgA, was chosen as a crude reflection of B-cell activity. Since we usually analyze a relatively large volume of samples in a day, it was apparent that many of the currently available techniques to quantitate specific serum proteins (i.e., radial immunodiffusion, radioimmunoassay, and electroimmunodiffusion) were unsuitable because of lack of automation. Another initial requisite, commercially available reagents, also was not feasible at that time; reagents were available to quantitate human serum immunoglobulins, but not for rats, dogs, and monkeys. We chose to produce monospecific, high avidity antibodies from goats directed against IgG, IgM and IgA from all three species and to use the nephelometric technology developed by Ritchie (5). This highly sensitive method is capable of detecting the microprecipitate formed from an antigen (IgG, IgM, or IgA)–antibody reaction. Other automated systems are becoming available and we are in the process of evaluating them for the purpose of quantitating serum immunoglobulins.

Lymphocyte Blastogenesis

Perhaps one of the most universal assays used in immunology today is the induction of lymphocyte proliferation with mitogens or specific proteins, also known as the transformation or blastogenic assay. Certain plant lectins, such as concanavalin (ConA) and phytohemagglutinin (PHA) selectively stimulate T-cells to undergo DNA synthesis in a polyclonal fashion. On the other hand, bacterial products, such as lipopolysaccharide, polyclonally stimulate B-cells (at least in the mouse). The search for a potent B-cell mitogen in other species is still continuing: *Mycoplasma neurolyticum* appears to be a B-cell mitogen in the rat (*6*).

From information available in the murine system we developed a blastogenic assay in the rat. In order to interface with our current standard

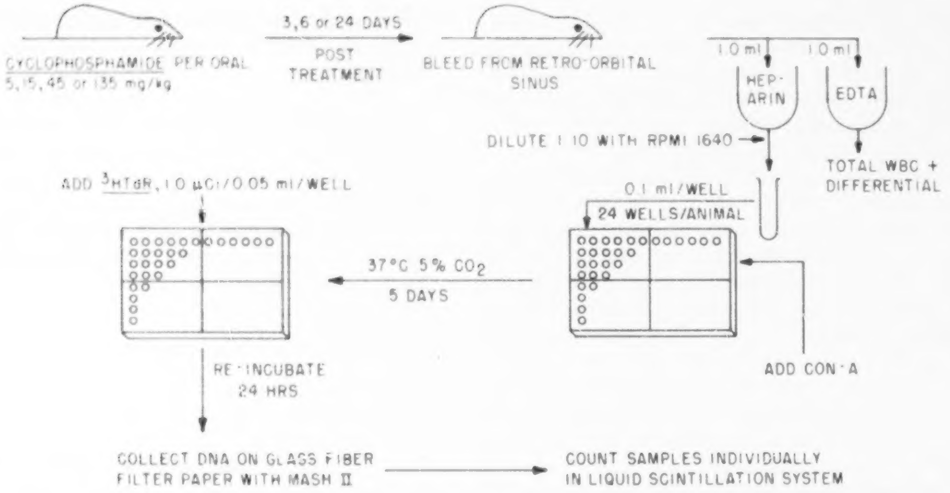
3,6 or 24 DAYS
POST TREATMENT
CYCLOPHOSPHAMIDE PER ORAL 5,15,45 or 135 mg/kg
BLEED FROM RETRO-ORBITAL SINUS
1.0 ml
1.0 ml
HEP-ARIN
EDTA
DILUTE 1:10 WITH RPMI 1640
TOTAL WBC + DIFFERENTIAL
ADD ³HTdR, 1.0 µCi/0.05 ml/WELL
0.1 ml/WELL
24 WELLS/ANIMAL
37°C 5% CO₂
5 DAYS
ADD CON-A
RE-INCUBATE 24 HRS
COLLECT DNA ON GLASS FIBER FILTER PAPER WITH MASH II
COUNT SAMPLES INDIVIDUALLY IN LIQUID SCINTILLATION SYSTEM

FIGURE 1. Protocol for the lymphoproliferative response of rat peripheral blood cells by mitogens.

operating procedures, we modified the whole blood assay described previously by Han and Pauly (*7*) (see Fig. 1). At the same time that the hemotologic sample was taken, a sample for the blastogenic assay was obtained. After a standard dilution, the cells were cultured with the appropriate mitogen and the functional activity of the respective populations of lymphocytes was assessed. During the course of development, we considered whether or not additional nutrients, such as fetal calf serum or mercaptoethanol, should be used to supplement the media. The addition of these supplements did not increase the magnitude of the responsiveness sufficiently to warrant their use (Norbury, unpublished findings). In addition, it was reasoned that by adding them, any immunopotentiating effect might be masked.

With this technique we were able to show immunosuppressive activity with cyclophosphamide (*8*) and dexamethasone but not with aspirin (Table 1).

Table 1. Mitogen-induced proliferative responses of rat peripheral blood cells *in vitro* following various treatments *in vivo*

Treatment group	Number of lymphocytes, % of control	Relative proliferation index[a]					
		Concanavalin A		Phytohemagglutinin		Pokeweed mitoge	
		(2-4 µg/well)	(0.32-0.64 µg/well)	(1.6-3.2 µg/well)	(6.4 µg/well)	(1.6-6.4 µg/well)	(1.6- 4 µg/w ll)
Purification by dextran-citrate[b]		−	+	−	+	−	+
Cyclophosphamide ($n = 8$)[c]							
10 mg/kg	81	0.59*	3.29	0.44*	1.50	0.70	1.4
50 mg/kg	32	0.02*	0.70	0.04*	0.48*	0.13*	0.3
Dexamethasone ($n = 8$)[d]							
0.35 mg/kg	43	0.08*	1.48	0.05*	0.88	0.17*	1.0
0.70 mg/kg	41	0.01*	1.68	0.96*	0.99	0.15*	1.7
Aspirin ($n = 5$)[e]							
100 mg/kg/day	111	0.94	NT	NT	NT	NT	N
200 mg/kg/day	123	0.84	1.96[f]	NT	NT	NT	N

*Significantly different from controls ($p \leq 0.05$) using anti-rankit transformation.

[a] Relative proliferation index is calculated from the following formula: mean counts per minute (treated)/mean counts per minute (controls) at the optimal concentrations (indicated in parentheses) for each mitogen. NT = not tested.

[b] Lymphocytes were collected from the buffy coat and plated at a concentration of 10^5 cells per well in 96-well microtiter plates.

[c] Animals were given a single oral dose; peripheral blood was collected from the retro-orbital plexus 4 days later.

[d] Animals were given a single oral dose; blood was collected 2 days later.

[e] Animals were dosed orally for 30 days and then bled.

[f] Lymphocytes were depleted of erythrocytes by ammonium chloride lysis.

An appealing feature of this assay is the fact that an antigenic stimulus of the animal is not required, thus eliminating the need to manipulate animals in a manner that may jeopardize the integrity of the routine toxicological evaluation. However, one important disadvantage of this technique is not knowing the fate of the lymphocyte once it is cultured. In the case of cyclophosphamide, a slight reduction (19%) in the number of circulating lymphocytes caused a significant suppression of the lymphoproliferative response to both ConA and PHA. At higher doses, cyclophosphamide caused marked lymphopenia with concomitant reduction in the lymphoproliferative response. It was entirely possible that the blastogenic assay was merely a reflection of the number of immunocompetent cells in culture. However, when the number of lymphocytes separated by dextran-citrate was adjusted to a constant concentration based on viability, the residual lymphocytes from cyclophosphamide-treated animals still had significantly impaired responsiveness to PHA (Table 1). Better techniques will be needed to determine if the lymphocytes from treated animals are still viable after 5 days in culture, especially when using a whole blood preparation.

Enumeration of Lymphocyte Subpopulations

In the event that an immune function test demonstrates impairment, it behooves the investigator to determine the number of immunocompetent cells. Lymphocyte subpopulations can generally be classified into three broad categories: T-cells, B-cells, and null cells. It is now known, particularly with the T-cell population, that a variety of subsets (T helper, T suppressor, T killer, etc.) exists and the means to quantitate them needs to be seriously considered. Once again, various methods are available, including immunofluorescence, rosette formation, histochemistry, cell electrophoresis, cytolysis and fluorescence-activated cell sorting. Immunofluorescence is the most popular technique, especially in working with rodents. The rosette formation assay appears to have some limitations when working with species other than human and is prone to interlaboratory variation. Histochemical methods also have limited application aside from human studies and cell electrophoresis is too analytical for immediate use. The complement-dependent, antibody-mediated cytolytic test can be used for quantitating lymphocyte subpopulations of lymphocytes on a routine basis. (I am not implying that none of the other methods can or will eventually be modified for routine testing.) With commercially available rabbit anti-rat thymocyte serum plus complement we obtained approximately 90% lysis of rat thymocytes and 50-55% lysis of splenic lymphocytes using the Coulter counter. The application of this particular technique to other peripheral organ sources (lymph node and peripheral blood) has not been as fruitful. Obviously much more work is needed in this area.

From this three-part program, the concept of a

Table 2. Special follow-up studies using common immune models.

	Study
Cell-mediated immunity	Memory (secondary antigen exposure)
	Lymphocyte mediated cytotoxicity
	Mixed lymphocyte reaction
	Lymphokine production
Humoral immunity	Antibody response to T-independent antigen
	Memory
	Plaque-forming cell quantitation
Reticuloendothelial system	
Resident/recruited alveolar and peritoneal cells	Number
	Phagocytosis
	Response to lymphokines
	Cytotoxicity
	Chemotaxis
	Enzymatic activity
Polymorphonuclear leukocytes	Mast cell degranulation
	Chemotaxis
	Phagocytosis
Other	Immunopathology
	Stem cell colony formation
	Complement activity
	Antinuclear antibodies

basic screen evolved to provide a minimum number of assays that would allow a meaningful interpretation of immunologic alterations. The basic screen includes the determination of total serum IgG, IgM and IgA, blastogenesis with T- and B-cell mitogens as well as specific antigens, cell enumeration, total leukocyte and differential, delayed type hypersensitivity, primary antibody responses to a T-dependent antigen, clearance of particulate matter by the reticuloendothelial system, and histopathology. This satisfies most of our initial criteria for evaluating T-cell and B-cell responses as well as macrophages while keeping the number of assays to a minimum. Such assays are relatively simple to perform and the technology now exists to implement them. However, it does appear that we may have to include additional animals that would not necessarily be processed as part of the routine toxicity study.

Until disproven, my bias is that the evaluation of antibody titers to a T-dependent antigen will eventually represent the best single analysis, closely simulating the immune response to infection. An antibody assay is quite sensitive and represents a response to a defined antigen involving the same types of cells that mediate immunity to infection.

In the event that the basic screening reveals alterations or inadvertent modifications of the immune response, special follow-up studies (Table 2) can be carried out to clarify the mechanism of action and the cell types involved, with particular emphasis on defining the subpopulations affected by the drug. Nevertheless, it is important to understand the limitations of any assay before making a judgment regarding the effect of a drug on the immune response.

Future of Immunotoxicology

In addition to on-line testing, there are other areas where immunology can be applied within the pharmaceutical industry.

Skin Sensitization

New developments in the methodology used to evaluate drug-induced hypersensitivity, or skin sensitization, has made it possible to improve the credibility of certain obligatory tests. The Landsteiner-Draize guinea pig skin test has long been the method of choice for doing skin sensitization. However, the development of the skin maximization test (*9*) has greatly improved the predictability of the guinea pig model while eliminating false negatives.

Development of Biologicals

Some pharmaceutical companies are actively developing viral vaccines as well as other adjuvants. Immunotoxicology would offer a dimension beyond that capable of routine toxicology for the assessment of potential adverse effects that might be immunologically mediated.

Development of Specific Immune Modulators

While the search for new and better anti-inflammatory compounds is an active program of many institutions, the toxicological examination of these new product candidates, inclusive of immune function testing, offers a broader basis for determining the potential of that particular drug.

New Product Development

The toxicologic testing of compounds that are not specifically designed as immune modulators may, in remote instances, demonstrate immunologic enhancement or impairment. Such information could then be referred back to medicinal chemists and pharmacologists for further development.

Basic Research

To aid in the development of more rapid and reliable assays as well as new immune models, which are critical to understanding the effects of chemicals on the immune response, some effort should be made in an immunotoxicology program to conduct basic research.

Conclusion

The field of immunotoxicology is in the early stages of rapid development. Although the immune response is quite complex, involving a sophisticated array of cellular and molecular interactions, there is increasing evidence that chronic, subclinical exposure to a variety of chemicals does in fact lead to an adverse modification of immune function, manifested most ostensibly by reduced host resistance to infectious agents, such as viruses and bacteria (*10*). In many respects, the state-of-the-art is much like mutagenesis was five to ten years ago. Yet we must realize that the progress of immunotoxicology will depend upon the continued, close interaction among persons in academia, industry, as well as governmental institutions. With the approach evolving at MSDRL, it now seems possible to move ahead in this area. Ultimately, a basic screen for immune competence needs to be validated using compounds with known immunosuppressive effects as part of a subacute study. In doing so, one must be prepared to explain any alteration in immune function in the absence of overt toxicity as typically described by physical observation, hematological and serum biochemical studies, urinalyses, and gross and histomorphologic examination. With further development, immunotoxicology can become part of the repertoire of toxicity testing, as we have seen for teratology and mutagenicity.

The author recognizes the excellent technical assistance rendered by Mr. C. Noble and wishes to thanks Ms. J. Arizin for typing the manuscript and Drs. J. S. MacDonald and A. E. Munson for their valuable comments.

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Environmental Health Perspectives
Vol. 43, pp. 61-69, 1982

Approaches to Assessing Host Resistance

by S. Gaylen Bradley* and Page S. Morahan*

There is increasing evidence that chronic, subclinical exposure to certain environmental pollutants may upset immune responsiveness and alter susceptibility of animals to infectious agents. Environmental chemicals or drugs may affect diverse aspects of the immune system, leading to immunosuppression, immunopotentiation, hypersensitivity or perturbed innate host resistance. A variety of infectious models is available that involves relatively well defined target organs and host defense mechanisms; for example, infections with encephalomyocarditis virus, *Herpesvirus simplex, Listeria monocytogenes, Streptococcus pneumoniae, Escherichia coli* or *Plasmodium berghei*. Important variables in infectious models used to assess immunotoxicity include species and strain of animal used, their age and sex, the route of exposure, and dose of the chemical. No one infectious model has yet emerged as a routine screening tool to detect and assess the subtle effects that may occur in immune responses when animals are exposed to doses of environmental pollutants that cause no adverse effect at a gross level. The selection of useful test systems is complicated because it is difficult to measure the effects of chronic, subclinical exposure to chemicals and sublethal challenges of microorganisms.

Introduction

Chronic, subclinical exposure to certain environmental pollutants or drugs may perturb immune responsiveness and alter susceptibility of animals to infectious agents. Environmental chemicals or drugs may affect diverse aspects of the immune system leading to immunosuppression, immunopotentiation, perturbed innate host resistance and hypersensitivity. A variety of experimental methods are being developed and evaluated in order to assess changes in host resistance following exposure to chemicals. Particular aspects of the vast array of cellular products and cell types involved in immunity can be examined selectively in appropriate models for monitoring host resistance in whole animals. Many of the complexities attendant to the effects of chemicals on immunologic processes, however, are unresolved. It is timely, therefore, to ask whether infectious models can be used routinely to assess immunotoxicity, what their reliability is, and whether they have utility in predicting human risk.

There is evidence that exposure to environmental pollutants causes alterations in various immune responses. Our colleagues have shown, for example, that polychlorinated biphenyls, chloroform and trichloroethylene administered orally to mice for 90 days lead to immunosuppression of some immune responses (*1-4*). In addition, Vos (*5*) and Koller (*6*) have reviewed some results with other chemicals. There is considerable difficulty in interpreting data from different laboratories however. This complexity arises in large part from the rapid proliferation of information and methodology in the field of immunology. Moreover, the protocols used to measure immune functions and the methods for calculating and reporting data may be quite dissimilar for different laboratories. In order to resolve this dilemma, it is necessary to develop a battery of standard assays that can be reproducibly used in different laboratories. Other reasons for inconsistency in results stem from the differences

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Table 1. Important variables in assessing host resistance.

Variable	Routine screening	In-depth study
Strain	One strain	Many strains
Sex	One sex	Both sexes
Age	Young adult	Weanling to aged
Exposure period	Subchronic – 14 days	Subchronic – 90 days
Exposure route	Intraperitoneal	Several routes
Exposure relative to challenge	Pre- and post-challenge	Only before or only after challenge

in animals used (species, strain), in sex, in age (neonatal, weanling, young adult, adult, aged) and finally differences in exposure to the chemical under study (administration before, during or both before and during challenge) (Table 1). In an in-depth study, it may be necessary to use a number of strains because of genetically determined differences. Male and female animals may vary in their handling of a chemical. Weanling or aged animals may be more susceptible to immunotoxicologic effects because such animals are somewhat immunodeficient. Long exposure periods may be necessary because definitive organ site toxicity may not be manifested within a shorter period, such as 14 days. Exposure by inhalation, oral administration or dermal contact may be a more appropriate model for assessing the effects of environmental exposure than intraperitoneal administration.

Effects of Chemicals on Infectious Organisms in Animals

A growing number of reports on adverse effects of environmental chemicals on host resistance to various infections in animals have appeared during the past decade. Some of these are summarized in Table 2. Virus infections, especially with encephalomyocarditis virus, have been the most studied. Interpretation of results from studies using infectious models is even more difficult than those using immunologic assays. Several species as well as different strains of animals have been used, in addition to male and female animals, different ages, different routes of exposure to the chemical (parenteral, oral), and different timing regimens (acute, chronic). These variables have also been confounded by using a variety of infectious agents, for which the pathogenesis and host resistance parameters have been fully established in only a few instances.

We have previously shown that treatment intraperitoneally with some immunomodulators changes host resistance to *Herpesvirus simplex* locally but has no effect systemically (*34*). Similar differences have been observed recently between local and systemic effects in mice fed a high fat diet and given immunomodulator intraperitoneally before Listeria infection. The liver and its macrophages proved to be the target most adversely affected by the high fat diet, while the fixed macrophages in the peritoneal cavity were normally activated after immunomodulator treatment (Campbell and Loria, unpublished results). These data emphasize the importance of separating local from systemic effects, and the necessity of using microbial infections for which the pathogenesis and immune responses have been well characterized. The standard toxicity parameters of lymphoid organ weight, and hematologic profiles are also important in interpreting any changes in host resistance properly.

Potentially Useful Viral Models

Infection with the picornavirus, encephalomyocarditis virus (EMC), has been used extensively in immunotoxicologic studies. This picornavirus produces a rapid systemic illness in mice, particularly in the heart and brain target organs. Systemic infection by EMC is one of the standard infections used to assess efficacy of antiviral therapy (*35*). Host defense mechanisms involved in recovery from this infection have been extensively documented; they include an early interferon response and induction of systemic neutralizing antibody which is independent of T-lymphocytes in the primary response (*36*, *37*). Studies on depletion of macrophages in infection with another picornavirus, Coxsackievirus, have indicated a role for these cells, possibly in an antibody-dependent cell-mediated cytotoxicity (ADCC) reaction which would amplify the viral specific antibody (*36*). Resistance to this virus is not markedly age related, but there are differences between the sexes in some mouse strains (*38*). Genetic resistance has not been as extensively studied as for some other virus infections. The primary importance of antibody in resistance to another picorna-

Table 2. Microbial infections used to assess changes in host resistance upon exposure of animals to environmental chemicals.

Microorganisms	Chemical	Animal	Reference
Viruses			
Herpesviruses			
Pseudorabies virus	Arsenicals	Mice	(*7*)
	TCDD	Mice, C57BL/6Jth	(*8*)
Herpesvirus simplex	Cannabis	Mice, BALB/c	(*9*)
	PCB	Mice, Swiss ICR	(*10*)
Enteroviruses			
Duck hepatitis virus	Dieldrin	Mallards	(*11*)
	DDT	Mallards	(*12*)
	PCB	Mallards	(*13*)
Encephalomyocarditis virus	Toximul MP88 and Atlox 3409 emulsifiers, DDT	Mice, Swiss ICR	(*14-17*)
	Arsenicals	Mice	(*7*)
	Cobalt sufate	Mice	(*18*)
	Cadmium, mercury, lead nickel	Mice, CD-1	(*19*)
	Methylmercury	Mice, Swiss-Webster	(*20*)
	Lead	Mice, Swiss-Webster	(*21*)
Arboviruses			
St. Louis encephalitis	Arsenicals	Mice	(*7*)
Eastern encephalitis virus	Arsenicals	Mice	(*7*)
Langat virus	Lead	Mice	(*22*)
Myxoviruses			
Influenza virus	Sulfur dioxide	Mice, Swiss-Webster	(*23*)
Retroviruses			
Moloney leukemia virus	PCB	Mice, BALB/c	(*24*)
Rauscher leukemia virus	Arsenicals	Mice	(*7*)
	Methylmercury	Mice, BALB/c	(*20*)
	Lead	Mice	(*25*)
Bacteria			
Salmonella bern	TCDD	Mice, C57BL/6Jth	(*8*)
Salmonella typhimurium	Lead	Mice, Swiss-Webster	(*26*)
Listeria monocytogenes	Cannabis	Mice, BALB/c	(*9*)
	PCB	Mice, Swiss ICR	(*10*)
Escherichia coli	Lead	Rat	(*27*)
	Cadmium	Mice	(*27*)
Staphylococcus epidermidis	Lead	Rat	(*27*)
Staphylococcus sp.	Sevin, Dicresyl, Jalan Tilliam, Maneb	Rat	(*28*)
Mycobacterium bovis	Cadmium	Mice, B10-A-2R	(*29*)
Parasites			
Hexamita muris	Cadmium	Mice, Swiss-Webster	(*30*)
Plasmodium berghei	PCB, HCB	Mice, BALB/c	(*31*)
Histomonas meleagridis	*p,p'*-DDT	Chickens	(*32*)
Heligmosomoides polygyrus bakeri	DDT	Mice	(*33*)

virus, echovirus, has also been well documented in patients with agammaglobulinemia (*39*).

Although treatment with an environmental contaminant may reduce various immune responses such as antibody production, it does not inevitably decrease host resistance. In an experiment in which mice were administered trichloromethane by gavage for 14 days, there was no significant change in the delayed hypersensitivity response to sheep erythrocytes nor was there a significant decrease in resistance to EMC virus (Table 3). Serum antibody titers to sheep erythrocytes however were decreased by 63% and 85% with doses of 50 and 250 mg/kg, respectively, in female mice, and by 70% and 79%, respectively, in male mice.

Another infectious agent that may prove useful in immunotoxicity is *Herpesvirus simplex* (HSV). We and others have clearly documented the multifaceted aspects of the immune response that may be involved in resistance to HSV infections (*40, 41*). Sensitization of T-lymphocytes, with subsequent activation of macrophages for antiviral activity, appears to be prominent in host resistance. Whether the same types of macrophages or macrophage functions are involved in antibacterial, antiviral and antitumor resistance is as yet unclear. We have shown that antiviral and antitumor functions of macrophages do not necessarily occur simultaneously (*42*). A role for interferon in the early stages of infection has been documented by

Table 3. Effects of trichloromethane on resistance to encephalomyocarditis (EMC) virus and the immune response to sheep erythrocytes (sRBC).[a]

Treatment, mg/kg	Anti-sRBC titer	Log LD_{50} (95% confidence limits)
None	3401 ± 403	7.2 (6.0–8.0)
Vehicle	5295 ± 808	6.9 (6.2–7.6)
5	4040 ± 318	6.7 (6.1–7.3)
50	1960 ± 234	7.5 (6.5–8.4)
250	820 ± 225	6.4 (6.0–6.9)

[a] Six-week-old female ICR mice were gavaged daily for 14 days with the indicated doses and then challenged intravenously with dilutions of encephalomyocarditis virus, and the LD_{50} determined. Mortality was measured over a 14-day period. Separate groups of mice were immunized with sheep erythrocytes on day 8 of exposure. Serum was collected on day 15 and serum antibody titers were determined. Each anti-sRBC value represents the mean ± standard error of the reciprocal of the titer, based upon 8 mice per group.

showing decreased resistance to HSV in mice treated with anti-interferon serum (*43*). Recently, a role for natural killer (NK) cells and for activated macrophages has been demonstrated for the early stages of infection (*44*). The induction of the T-cell-dependent neutralizing antibody does not appear to be of primary importance in the initial reduction of virus, but may be involved in recovery and altering the latent infection with HSV which often follows primary infection (*45*). Very small amounts of antibody may be amplified tremendously (several magnitudes) by the ADCC reaction (*46*); thus antibody in conjunction with mononuclear phagocytes may also be involved in recovery from primary infection. The presence of neutralizing antibody is sufficient to protect mice from a second challenge with the virus (*47*). HSV infection will thus monitor the adequate functioning of many elements in the immune system: interferon induction, T-lymphocytes, B-lymphocytes, NK cells and macrophages. For these reasons, we believe that HSV infection may be a very sensitive indicator of changes in any aspect of the immune system. We have shown, for example, that depression of either macrophage function or T-lymphocytes markedly reduces resistance to this virus (*40*).

Another potential advantage of this virus infection is that different pathogeneses of infection and induction of immune responses occur with different routes of inoculation (*47*, *48*). A local infection resulting in vesicles can be observed clinically after labial or vaginal inoculation of HSV. Moreover, HSV often becomes latent in the sensory ganglia in survivors of infection and the incidence of latency can be markedly altered by changes in the immune responses of the animal (*45*). The latent virus can be reactivated by immunosuppression (*49*). The labial infection is particularly advantageous in all of these respects (local infection, systemic immune responses, latency in the easily accessible trigeminal ganglion, alteration of latency by immune responses and reactivation of latent virus).

There is clear evidence for genetic resistance to HSV. C57BL/6 mice are among the most resistant, and resistance is dominant (*50*). The mechanism of resistance has not been completely defined; Lopez's present hypothesis is that a marrow dependent cell, possibly the NK cell, is involved. We and others have also documented that resistance to HSV also increases considerably with the age of mice (*40*).

Potentially Useful Bacterial Models

Infection with the gram-positive bacterium *Listeria monocytogenes* has been extensively investigated in regard to pathogenesis of infection, and the immune responses that are involved in recovery of animals from infection. From the data reported in the series of elegant studies of Mackaness and colleagues at the Trudeau Institute (*51*, *52*), it is now well accepted that recovery from infection depends upon specific sensitization of T-lymphocytes that then activate macrophages for enhanced nonspecific bactericidal activity. Fixed macrophages are also involved in the initial inactivation of the bacterium, while the activation of macrophages occurs within 2-3 days following systemic infection. Resistance to the bacterium can be assessed by mortality, or by bacterial colony counts of the liver and spleen, which are major sites for replication of the bacteria.

The resistance of mice to Listeria can be profoundly altered by the genetic makeup of the animal. For example, the C57BL/6 mouse is resistant, while the A mouse is susceptible (*53*). The mechanism of the genetic resistance is as yet unclear. Bone marrow studies have indicated that resistance is determined by the host rather than by the donor, and that the spleen of the resistant animal is required for expression of the genetic resistance. The current hypothesis is that genetic resistance involves an interaction between macrophages and the microenvironment provided by the resistant genotype (*54*). Despite the complexity of these interactions, Listeria infection provides a reliable model that has been well characterized. The infection primarily assesses competency of T-lymphocytes and macrophages.

Table 4. Effects of cannabinoids on resistance to ***Herpesvirus simplex*** **type 2 (HSV-2) and** ***Listeria monocytogenes*** **infections.**

Treatment[a]		Decrease in resistance to[b]	
Drug	Dose, mg/kg	Listeria	HSV-2
Vehicle	---	2	6
Δ^9-THC	200	365[c]	96[c]
Marijuana extract	200	88[c]	3
Flumethazone	5	385[c]	10[c]
Cyclophosphamide	200	Not done	260[c]

[a] Mice were inoculated intravenously with dilutions of each microorganism and treated intraperitoneally on days 1 and 2 with the indicated doses of drug. The LD_{50} for HSV-2 was $10^{1.9}$ (95% confidence limits $10^{1.4}$ to $10^{2.4}$), expressed as dilution factors. The LD_{50} for Listeria was $10^{5.2}$ (95% confidence limits $10^{4.8}$ to $10^{5.5}$) colony-forming units. See Morahan et al. (*9*) for complete data.

[b] Decrease in resistance: LD_{50} for untreated group/LD_{50} for treated group.

[c] Significantly different from the untreated group at $p < 0.05$.

Table 5. Effects of polychlorinated biphenyl (PCB) on resistance to ***Listeria monocytogenes*** **or** ***Herpesvirus simplex*** **type 2 (HSV-2) infections.**

Treatment[a]		Decrease in resistance to[b]	
Drug	Dose, mg/kg	Listeria	HSV-2
PCB vehicle		1	1
PCB	30	3	1
Flumethazone vehicle		2	1
Flumethazone	5	794[c]	20[c]

[a] BALB/c mice were treated for 14 days by gavage with PCB prior to infection or for two days after infection by intraperitoneal administration of flumethazone. Mice were infected intravenously with dilutions of microorganisms. The LD_{50} for HSV-2 was $10^{2.3}$ (dilution factor) and for Listeria the LD_{50} was $10^{3.4}$ colony-forming units. These data were kindly supplied by Munson et al. (*10*).

[b] Decrease in resistance: LD_{50} for untreated group/LD_{50} for treated group.

[c] Significantly different from the untreated control at $p < 0.05$.

We have used both Listeria and HSV infections to assess host response to environmental toxicants and drugs. In one study, the effects of treatment with marijuana extract or with the pure chemical Δ^9-tetrahydrocannabinol (Δ^9-THC) was compared with effects of treatment with the known immunosuppressive alkylating agent, cyclophosphamide, or the steroid flumethazone (Table 4). The standard immunosuppressive regimens markedly decreased resistance to both HSV and Listeria, as did treatment of mice with Δ^9-THC. The course of the Listeria infection was also exacerbated by treatment with marijuana extract but the HSV infection was not. Although there are certain similarities in resistance mechanisms to these two microorganisms, it is clear that there are differences, too (*9*).

In another study, mice were exposed by gavage to polychlorinated biphenyls (Aroclor 1254) daily for 14 days prior to infection with Listeria or HSV-2 (Table 5). Whereas treatment with the known immunosuppressive steroid flumethazone decreased resistance significantly, there was no effect with the polychlorinated biphenyl exposure. This lack of effect on host resistance contrasts with the marked effect observed with this environmental toxicant on standard immune assays. Serum antibody titers to sheep erythrocytes were reduced dose dependently to 20% of control values by 2[illegible] mg/kg in male mice and to < 1% of control values in female mice. The delayed hypersensitivity response to sheep erythrocytes was completely abolished in male mice only with the highest dose tested (75 mg/kg); however, a dose of 12.5 mg/kg reduced the delayed hypersensitivity response by 40%. Flumethazone at 5 mg/kg administered subcutaneously on days 1, 2 and 3 after antigen completely suppressed both humoral and cell mediated responses to sheep erythrocytes.

The pathogenesis and immune responses to *Streptococcus pneumoniae* infection have been extensively characterized also. Recovery from infection depends upon the induction of opsonizing antibody, which in conjunction with phagocytic cells, enhances phagocytosis of *S. pneumoniae* and its subsequent intracellular destruction (*55-57*). End points of mortality and measurement of antibody status (by *in vitro* methods or rechallenge of the surviving animals) can be determined. This classic infection thus measures the function of B-lymphocytes and plasma cells to produce the T-cell independent antibody to the pneumococcal polysaccharide, as well as the functional capacity of granulocytic, phagocytic cells.

The gram-negative enteric bacterium *Escherichia coli* is capable of causing a variety of extraintestinal infections in man. Virulence of different strains in intraperitoneal infection of mice correlates well with virulence in natural infection (*58*). Mouse virulence is directly related to survival and multiplication in the peritoneal cavity leading to production of large amounts of endotoxin (*58, 59*). The virulence is correlated with resistance to phagocytosis by both macrophages and polymorphonuclear phagocytes (*58, 59*). Phagocytosis can be enhanced by the presence of opsonins and complement in normal serum or specific antibody to *E. coli* K antigens (*58, 60*). In the later phases of infection, survival should be dependent on the animal's defense against the effects of endotoxin. The

Table 6. Effects of chemicals on resistance to live *Escherichia coli* and purified endotoxin.

Treatment[a]		Decrease in resistance to[b]	
Chemical	Dose, mg/kg	*E. coli* cells	Endotoxin
None	---	1	1
Actinomycin D	0.4	55[c]	266[c]
6-Mercaptopurine	100	55[c]	23[c]
Δ^9-Tetrahydrocannabinol	150	7[c]	12[c]
Vincristine	1	9[c]	13[c]

[a] Male mice were injected intraperitoneally with drug, live *E. coli* cells or purified gram-negative bacterial endotoxin as indicated. The LD_{50} for actinomycin D was 0.8 mg/kg; for 6-mercaptopurine, 220 mg/kg; for Δ^9-tetrahydrocannabinol, 350 mg/kg; for vincristine, 7 mg/kg; for live *E. coli*, 5.5×10^9 cells/kg; and for purified *E. coli* 0127:B8 (Boivin) endotoxin, 16 mg/kg.

[b] Decrease in resistance: LD_{50} for untreated group/LD_{50} for treated group.

[c] Significantly different from the untreated group at $p < 0.05$.

extraintestinal virulence of a particular *E. coli* strain for mice is influenced by the presence and identity of O and K surface antigens, especially Kl (*60*).

The susceptibility of animals to gram-negative bacteria and their endotoxins can be altered dramatically by a variety of chemicals (*61*). We have studied extensively the enhanced toxicity of gram-negative bacteria or endotoxin in combination with drugs for more than a decade. Drugs that inhibit protein synthesis or ribonucleic acid synthesis render animals unusually susceptible to live *E. coli* or purified endotoxin whereas endotoxin renders animals particularly vulnerable to the adverse effects of drugs that affect deoxyribonucleic acid synthesis, structure or function (*61-63*). Actinomycin D, 6-mercaptopurine, Δ^9-THC and vincristine, for example, at sublethal doses impaired the resistance of mice to challenge with live *E. coli* or purified endotoxin (Table 6). The extent of the decrease in resistance with respect to the two endotoxic sources was similar for each chemical, affirming that endotoxin has some role in the pathogenesis of *E. coli* infections. The interlock among the actions of the host on the drug, of the bacterial pathogen and the test chemical on the host, and of the chemical on the bacteria are especially complex in *E. coli* infections (*62*). Bacterial endotoxin (purified or as gram-negative cells) impairs the activity of the liver mixed function oxidases in treated animals. Several chemicals, for example chlorambucil, are metabolized by these microsomal enzymes (*61-63*). Elevated drug levels resulting from impaired detoxification may retard the growth of live *E. coli* if the test chemical has antimicrobial activity, or they may exacerbate the endotoxic action of the gram-negative bacterium by rendering the host hyperreactive to endotoxin. A number of chemicals affect the integrity of the intestinal mucosa, thereby allowing the "endogenous" gram-negative bacteria to gain access into the circulatory system. These interconnected events may establish a cycle of adverse effects that amplify the interaction between a chemical toxicant and an infectious agent.

Potentially Useful Protozoan Model

Plasmodium berghei, an intracellular sporozoan parasite causing malaria in a variety of rodents, is potentially useful in immunotoxicity assessments (*64*). Mice and rats infected with *p. berghei* have been used extensively as models for examining immunity and host defense mechanisms in malaria and as *in vivo* assays for potential antimalarial drugs. With highly adapted parasites such as Plasmodium, a combination of immunological effector mechanisms of host resistance is well recognized. Ingestion and destruction of antibody-coated or surface-modified Plasmodium and Plasmodium-infected erythrocytes (by phagocytes including macrophages) is thought to be a common effector mechanism (*65, 66*).

More information is needed however on the molecular changes in parasitized erythrocytes at various stages of Plasmodium development and of the quantitative aspects of antigenic changes (and effects of antibody binding). Inhibition of invasion of erythrocytes by Plasmodium merozoites can be mediated by antibody (*67*). Evidence that T-lymphocytes may also be involved in resistance comes from the observation that mediators of nonspecific resistance, such as BCG organisms, are capable of affecting parasite elimination in some strains of mice while nude mice remain highly susceptible (*68*).

Conclusions

A variety of infectious models are available that involve relatively well defined target organs and host defense mechanisms (Table 7). It appears, however, that no one infectious model has yet emerged as a routine screening tool to detect and assess the subtle toxic effects that may occur in immune responses when animals are exposed to doses of environmental pollutants that cause no adverse effect at a gross level. This conservative judgement of the current state-of-the-art reflects both the sophisticated nature of the cellular and

Table 7. Synopsis of selected infectious models potentially useful in immunotoxicity assessments.

Organism	Target organs after ID or IV inoculation	Host defense mechanisms assessed primarily
Viruses		
Encephalomyocarditis virus	Heart, brain	Interferon production Antibody production by B-cells
Herpesvirus simplex	Local site of infection Central nervous system	Interferon production T-cells Macrophages Natural killer cells Antibody production by B-cells ADCC reaction[a]
Bacteria		
Listeria monocytogenes	Liver, spleen	T-cells Macrophages
Streptococcus pneumoniae	Spleen, liver, lungs, kidneys	Opsonizing (T-cell independent) antibody production by B-cells Antibody dependent phagocytosis and intracellular destruction by granulocytes
Escherichia coli	Peritoneal cavity, liver, spleen	Sensitization to endotoxin Phagocytosis and intracellular destruction by phagocytes Opsonizing antibody production by B-cells Complement
Parasites		
Plasmodium berghei	Erythrocytes, liver, spleen	T-cell dependent antibody production by B-cells Macrophages (ADCC reaction?)

[a] Antibody-dependent cell-mediated cytotoxicity.

molecular interactions operant in host resistance and the experimental reality that the optimum conditions for achieving sensitive and reproducible predictor systems in immunotoxicology have not been adequately defined. The selection of useful test systems will not be achieved easily because it is difficult to work with models concerned with the effects of chronic, subclinical exposure to chemicals. This difficulty is compounded when there is also a need to assess these effects in infection initiated with sublethal challenges of microorganisms.

It is timely to ask whether infectious models should be used routinely to assess immunotoxicity, and which immune dysfunctions cause increased risk to infectious agents or tumors in humans. There is no doubt that immunodeficiency in humans increases risk to infectious diseases. In general terms, patients with B-cell deficiencies are very susceptible to bacterial pathogens but have relatively normal resistance to viral infections. Patients with T-cell deficiencies are very susceptible to fungal, protozoan and viral infections and show increased vulnerability to intracellular bacterial parasites. Patients with phagocytic dysfunction are unusually susceptible to bacterial infections but have relatively normal resistance to viral or protozoan infections. In light of the increasing evidence that low levels of environmental pollutants affect the immune system and impair host resistance to infectious agents (*69*), it is prudent to evaluate the utility of selected infectious models in immunotoxicity assessment.

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Environmental Health Perspectives
Vol. 43, pp. 71-79, 1982

Use of Viral Infections in Animal Models to Assess Changes in the Immune System

by Earl R. Kern*

Viral infections in animal models appear to be ideal systems for determining toxicity to the immune system by environmental substances. Since many viral infections that are utilized in animals produce systemic disease, these models provide an opportunity to evaluate the interaction between virus and components of host resistance. In these infections it is possible to delineate the role of antibody, interferon, cell-mediated immunity, neutrophils and macrophages in response to infection. A change in any of these components responsible for resistance to a particular virus may be correlated with an alteration of mortality and pathogenesis of the viral infection. Three experimental viral infections in mice that are potential candidates for use in determining immunotoxicity are discussed in terms of the response of individual components of resistance to infection and how changes in these components result in alterations of viral pathogenesis. The resistance to encephalomyocarditis virus infection in mice appears to be primarily mediated by antibody and interferon while with herpes simplex virus, infections are mainly controlled through cell-mediated immunity, macrophages, and possibly interferon. Cellular immunity also appears to be primarily responsible for resistance to cytomegalovirus infections. Therefore, it is important in the use of these systems for evaluating immunotoxicity to define the pathogenesis of the viral infection and the specific host responses to these infections and to be able to correlate a change in host resistance with an alteration of the viral infection.

Interactions between Viruses and Host Defenses

Viral infections are probably the most common cause of morbidity in man. We are continually being exposed to a variety of different viruses, often become infected, but generally recover without serious consequences. There are a number of viruses, however, that do have the capability of producing severe disease in man and in many cases result in mortality. There are many factors that may play a role in determining whether the infected host recovers or not; the two most important appear to be the virulence of the virus and its pathogenesis of infection and the ability of the host's defense mechanisms to control the viral infection. This is a very complex set of interactions that result in disease manifestations ranging from complete recovery to death.

Once the host becomes infected, dependent on the particular virus, there are a variety of possible patterns of pathogenesis of the viral infection that may occur in the host. There may be disease only at the local site of infection such as the upper respiratory tract. An example of this type of infection is caused by rhinoviruses or other viruses which produce a limited respiratory infection. After infection at a local site there may be direct spread of virus to other target organs such as the lower respiratory tract. Influenza virus, the parainfluenza viruses and respiratory syncytical virus can result in this type of pathogenesis. The virus may spread from an

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initial site of infection to other target organs such as the lungs, liver, spleen, brain, or skin by way of the blood stream or the lymphatic system. The virus may be free in the plasma, be carried within leukocytes, or in some cases may actually replicate in the leukocytes. Some of the viruses that are disseminated through the host in this manner include measles virus, mumps virus, hepatitis virus, poliovirus, and the arthropod-born togaviruses. Certain viruses such as herpes simplex virus and rabies virus have the capability of traveling from an initial site of infection to the central nervous system by way of direct neural spread. The nature of the interaction between the infecting virus and the host's immune system is going to be dependent on the pathogenesis of the viral infection. The more generalized the infection is, the greater may be the role of the host's defense in contributing to the control of the infection.

There are numerous components of host resistance, both humoral and cellular that may respond to viral infections. These include antibody produced by B-lymphocytes, interferon, cytotoxic T-cells, natural killer (NK) cells, neutrophils and macrophages. In addition to these there are also subsets of some of these, particularly the T-lymphocytes, that carry out different functions. Some subsets may act as helper cells, while others may actually suppress certain immune functions. In addition to these individual components of host resistance, there are multiple interactions between the components. It would appear unlikely that one component of the immune system is responsible for the recovery of the host from a viral infection but rather that host resistance is a complex interaction of many components. There is also selectivity in that some components appear to be more important in recovery to certain viral infections than in others.

The interaction between the virus and host can result in one of a number of potential outcomes (*1*). These are summarized in Table 1. The first possibility is that the host is completely resistant and no infection is established. In this case other factors than just immune functions are undoubtedly involved. Secondly, the host may undergo a mild or subclinical infection with either a complete recovery or recovery with a persistent or latent infection. The third possibility is that the host will suffer from an acute disease. Depending upon the particular virus and the ability of the host's immune system to control the infection, the outcome can be death, complete recovery, or again recovery with a persistent or latent infection. Finally, the virus can produce a persistent infection which may be asymptomatic, may be characterized by periodic recurrences, or may continue as a long term persistent or chronic infection. The final determination as to whether most viral infections result in recovery, a chronic infection or death is probably mediated by the host's immune system. It is well documented that many of the severe or chronic infections occur in patients with compromised immune functions. The outcome of a particular viral infection, thus, reflects both the virulence of the virus and the immunocompetence of the host. Factors which compromise host defense mechanisms would be expected to alter the virus-host relationships, therefore, animal model infections may be utilized for evaluating immunotoxicity.

Table 1. Potential results of virus-host interactions.

Interaction	Result
Host is resistant	No infection established
Subclinical infection	Complete recovery Persistent or latent infection
Acute clinical disease	Death Complete recovery Recovery with persistent or latent infection
Chronic infection	Asymptomatic with latent virus Latent with recurrence Persistent-chronic infection Malignant transformation

If one is to use the immune system as a target organ for assessing the effect of environmental substances, it is necessary to understand how a change in the immune system might result in a change in the pathogenesis of the viral infection. A few examples of how an alteration in some of the components of host resistance might affect viral replication are shown in Table 2. A suppression in the synthesis or function of antibody, interferon, T-cells or macrophages would likely result in enhanced viral replication and dissemination which we would predict would result in more serious disease or enhanced mortality. On the other hand, if the substance happens to induce interferon or activate macrophages we would predict that in some infections viral replication would be decreased resulting in less serious disease and lower mortality. It is

Table 2. Predicted change in viral pathogenesis resulting from an alteration in components of host resistance.

Component	Change	Viral replication
Antibody	Depression	No change, increase
Interferon	Induction	Decrease
	Suppression	Increase
T-Cells	Suppression	Increase
Macrophage	Activation	Decrease
	Suppression or destruction	Increase

obvious from the vast information collected in both man and experimental animals that some components of the immune system can be modulated in either direction. Specific examples of how those changes can result in the alteration of viral infections in animals models will be considered subsequently.

Use of Animal Models to Assess Changes in Host Resistance to Viral Infections

In the development and use of viral infections in animal models for determining the toxicity of various environmental substances a number of concepts concerning the virus-host relationship need to be considered. Some of the "ideal" properties of viral infections in animal models are: the viral infection should simulate a relatively common human disease; whenever possible the animals should be infected by a natural route, preferably by the same manner in which man becomes infected; a low inoculum of virus should be utilized so that one does not overwhelm the host's immune system; human viruses or ones closely related should be utilized; the course and pathogenesis of infection should be similar to that seen in the corresponding disease in man; and the host used should have an immune response similar to that of man. It is probably not possible to fulfill all these criteria, however, we should strive to use as many of the properties as possible so that data collected in these models will be relatively predictive for what we would expect in man. Due to convenience and cost as well as viral susceptibility, most animal models have utilized mice. Although this host is a good one for immunological studies, its small size presents major problems for toxicological evaluation. Additionally, there may also be differences in metabolism, pharmacokinetics, etc., of the test substance.

The use of viral infections in animal models to determine changes in the immune system is ideal because an effect can be evaluated and correlated both in the viral infection and in the host's immune system. The most useful parameters that are used to determine changes in the viral infection are final mortality rates, mean survival times, viral replication in specific target organs, viral replication at local sites of infection and mean lesion scores, time to healing, etc.

Most commonly used—and, of course, the easiest parameter to use—is effect on mortality. Is the mortality rate enhanced or is the animal protected, and does the time period of survival change. Additional and often more important information can be gained by determining the effect on viral replication in specific target organs such as the lung, liver, spleen, brain or in the nasopharynx, genital tract, skin lesions, etc. These parameters are generally more sensitive in determining effects on the viral infection than is final mortality. There are numerous parameters which can be utilized to determine alterations of each component of the immune system. A partial list of immune functions that could be used are tabulated in Table 3. One approach would be to begin with two or three general tests, then proceed to the use of more specific functional assays. The major pitfall in the use of these *in vitro* tests is that they may not necessarily be predictive of the response in the intact animal. Additionally, it is difficult to evaluate noted changes in an isolated component when in the intact animal there is certainly interaction between the various components along with control mechanisms. If one is able to correlate changes in the immune system with an alteration in the pathogenesis of the viral infection, however, valuable information concerning the potential toxicity of environmental substances can be gained.

Table 3. Parameters used to determine alteration of host resistance: the immune system.

Immune function	Test system
Antibody response	Specific viral antigen Other antigens, SRBC, etc.
Interferon response	Viral infection Other inducers
Lymphocyte blastogenesis	Nonspecific mitogens Virus-infected cells
Lymphocyte cytotoxicity	
T-Cells	Virus-infected cells
NK Cells	Tumor cells
Macrophage function	Viral replication Phagocytosis, killing, etc. Cytotoxic for tumor cells Protect virus-infected cell Monolayer
Neutrophil function	Chemotaxis, phagocytosis Chemiluminescence

Potential Model Viral Infections for Evaluating Immunotoxicity

From the numerous experimental viral infections of animals available, I have selected three as examples of systems that might be used for determining effects on the immune system. These models were

Table 4. Potential model systemic viral infections.

Infection	Parameters
Encephalomyocarditis (EMC) virus infection of mice	Pathogenesis of disease Role of antibody Role of interferon
Herpes simplex virus (HSV) infection of mice	Pathogenesis Role of cellular immunity – immunosuppression Role of macrophage – suppression or activation Role of interferon
Murine cytomegalovirus (MCMV) infection of mice	Pathogenesis Role of cellular immunity – immunosuppression Alteration of host resistance by infection

selected not necessarily because they are the best but because there is a considerable amount of information available on the pathogenesis of the viral infections and on the role of the immune system in the control of these infections. The three viruses are relatively easy to work with and do not have a significant health hazard when used under reasonable laboratory conditions. These three model infections and some of the virological and host resistance parameters that have been evaluated in each system are shown in Table 4. For each of the model infections the pathogenesis and the role of some of the key components of host resistance in the control of the infection will be presented.

Encephalomyocarditis (EMC) Virus Infection of Mice

This experimental system is a model of human enterovirus infections caused by the Echo viruses, Coxsackie viruses, and polioviruses. After intraperitoneal (IP) or intranasal inoculation of mice with EMC virus, the pathogenesis of this infection is characterized by an initial viremia followed by replication of virus in the heart and then the brain (*2-4*). The animals generally die on days 6-8. Clearance of virus from the blood is associated with an early rise in interferon in the serum followed by antibody production. Therefore, both of these components of host resistance appeared to be involved in recovery from this infection. To determine the role of antibody, Murphy and Glasgow (*5*) x-irradiated mice prior to infection with a sublethal dose of EMC virus (Table 5). Either EMC virus infection or x-irradiation alone resulted in only a low mortality, whereas the combination of the two resulted in a 90% mortality, and no antibody could be detected in these animals. In contrast, however, when the x-irradiated animals infected with EMC virus were given passive antibody early in the course of the infection, most of the mice were then protected. These data indicate that the production of antibody by B-cells is certainly involved in recovery from this infection.

Table 5. Effect of administration of anti-EMC antibody on mortality of x-irradiated, EMC virus-infected mice.[a]

Treatment	Mortality, %
EMC virus only	12
x-Irradiation only	5
EMC + x-irradiation	90
EMC + x-irradiation + antibody at	90
–24 hr	0
+24 hr	20
+48 hr	35
+72 hr	55
+96 hr	80

[a] Modified from Murphy and Glasgow (*5*).

To evaluate the role of interferon in the EMC virus infection, two types of experiments have been performed. In the first case mice were inoculated with EMC virus and then given either passive interferon or poly I:C, which is an inducer of interferon, to determine if enhancing the amount of circulating interferon might result in the protection of the infected animals. In the second case, mice inoculated with EMC virus were given anti-interferon antibody to determine if elimination of interferon would result in the enhancement of mortality. The effect of treatment with passive interferon or poly I:C on the mortality of mice inoculated with EMC virus is presented in Table 6. The untreated control mice had a final mortality of 83%, while those treated shortly after infection either with interferon or the inducer were protected. Protection of these animals was associated with a significant alteration in the pathogenesis of the infection. In treated animals there was no detectable viremia and no seeding of the heart or brain (*3,4*). The effect of anti-interferon antibody on EMC virus infection of mice is shown in Table 7. When mice were inocu-

Table 6. **Effect of treatment with exogenous interferon or poly I:C on mortality of mice inoculated with EMC virus.**

Treatment	Mortality		p value
	n/tot	%	
None	33/40	83	—
Exogenous interferon			
-1 hr	0/15	0	< 0.001
+1 hr	2/15	13	< 0.001
+24 hr	11/15	73	N.S.
Poly I:C			
-6 hr	1/25	4	< 0.001
+1 hr	1/24	4	< 0.001
+24 hr	10/25	40	< 0.01

Table 7. **Effect of anti-interferon globulin on EMC virus infection of mice.**[a]

Virus dilution	Treatment	Mortality, %
10^{-6}	None	100
	Normal globulin	100
	Anti-IFN globulin	100[b]
10^{-7}	None	0
	Normal globulin	15
	Anti-IFN globulin	100[b]
10^{-8}	None	0
	Normal globulin	0
	Anti-IFN globulin	80

[a] Modified from Gresser et al. (*6*).

[b] Mice treated with anti-interferon globulin died 3-5 days earlier than untreated mice.

lated with a concentration of virus that resulted in 100% mortality, the ones treated with antibody to interferon died 3-5 days earlier than untreated ones. If the virus inoculum was reduced to where no control animals died, the administration of anti-interferon antibody resulted in 100% mortality. Additionally, the enhanced mortality in antibody-treated mice was correlated with a dramatic increase in viral replication in critical target organs (*6*). These data strongly suggest that interferon is also involved in host resistant to this type of viral infection. Enhancement of interferon production results in protection of the animal, while inhibition of the interferon response results in a marked enhancement of the infection. In experimental EMC virus and other similar enterovirus infections both antibody and interferon appear to be primarily responsible for resistance. Cellular-mediated responses appear to be less important.

Herpes Simplex Virus (HSV) Infection of Mice

Herpes simplex virus is the causative agent of a variety of diseases in man including herpes labialis, herpes genitalis, encephalitis, and disseminated infection in the neonate. There are two major antigenic types. The type 1 strains are generally associated with infections of the mouth, face and the central nervous system in adults and the type 2 strains which generally are responsible for genital infections and disseminated disease in neonates. A model systemic infection may readily be established in adult mice by IP administration of most type 2 strains; however, they are considerably more resistant to type 1 strains. In most cases, immunosuppressive agents need to be used prior to IP challenge with type 1 strains. For localized infections of the orofacial or genital areas either types can be used. Since the role of the immune system in localized HSV infection remains poorly defined this discussion will be limited to the use of systemic infections in mice. When mice are inoculated with HSV-2, the pathogenesis of the infection can be variable depending on the age of the animal and the route of viral inoculation. When three-week-old female mice are inoculated IP, virus is first detected in the gut, liver and spleen, and later on in the lung, brain and spinal cord. Virus is sporadically recovered from the blood. If a more natural route of infection is used by inoculating mice intranasally with HSV-2, virus is first detectable in the lung, with subsequent spread of virus through the blood to the liver and spleen. Concomitantly, virus also spreads from the nasopharynx by way of trigeminal nerves to the brain (*7*). In both of these models there is a generalized infection involving multiple target organs, particularly those containing the cellular components of host resistance such as the liver and the spleen.

Since most people who have recurrent HSV infections of the oral or genital regions also have high levels of circulating antibody, this component of immunity is probably not a major factor in host resistance to this virus. However, it has been demonstrated that the presence of antibody can prevent reinfection or result in less severe disease. Members of the herpesvirus group, including HSV, cytomegalovirus and varicella-zoster virus, frequently cause severe disease in immunocompromised patients, suggesting that cell-mediated immune functions may be the most important part of host resistance to these viruses. As mentioned previously, adult mice are highly resistant to infections with HSV-1. In the animal model, the use of immunosuppressive agents such as cyclophosphamide or anti-lymphocyte serum which destroys T-lymphocytes dramatically increase the susceptibility of animals to HSV (*8,9*). One representative experiment in which susceptibility of mice to HSV-1 given either orally or IP is shown in Table 8. Increased mortality was associated with enhanced viral replication in target organs

Table 8. Effect of cyclophosphamide (CPA) immunosuppression on mortality of herpes simplex virus (HSV) type 1 infection in adult mice.[a]

Treatment	Mortality	
	n/tot	%
HSV (IP) control	19/40	48
HSV (IP) + CPA	29/30	97
HSV (oral) control	1/30	3
HSV (oral) + CPA	13/30	43

[a]From Rajcani et al. (*10*).

(*10*). From the data available it seems certain that T-cells are involved in resistance to HSV.

Another component that appears to be an important determinant in resistance to HSV is the macrophage. These cells appear to be of major importance in the development of age-related resistance in that it has been shown that HSV will replicate in macrophages from susceptible newborn animals, but not in macrophages from resistant adult mice (*11,12*). Two general types of experiments further document the role of the macrophage in resistance to HSV. Agents such as silica which transiently destroy macrophages can result in enhanced susceptibility. The effect of silica treatment on the mortality of mice subsequently inoculated IP with HSV-2 is shown in Table 9. In two experiments with two different silica preparations, mortality was enhanced significantly compared to control animals. The increase in mortality was correlated with a change in the pathogenesis of the HSV-2 infection. In control mice, low levels of virus were found sporadically in the visceral organs, and virus was not detected in the brain until late in the course of infection. In contrast, in silica-treated mice, virus was present in high titers in target organs 2 days after infection, and virus was detected in the brain on day 4 (*13*). The second concept that can be used to implicate the macrophage as a primary component of host resistance to HSV comes from the fact that immunomodulators such as BCG, *C. parvum* and pyran which have been shown to activate the macrophages or increase their function result in increased resistance to HSV infection (*13-15*). An example of the use of these substances to increase resistance to HSV-2 is shown in Table 10. To be effective in increasing resistance the substances must be given 5-14 days prior to infection. In these experiments the enhanced protection also correlated with the absence of virus in the target organs, and with the activation of macrophages by these agents. Therefore, resistance to HSV infection can be modulated by manipulation of the macrophage population. Destruction or suppression of macrophages results in increased susceptibility, while enhancement of macrophage activity results in increased resistance.

Table 9. Effect of silica treatment on systemic HSV-2 infection.[a]

	Log LD_{50}	
	Normal mice	Silica-treated mice
Experiment 1		
Sargeant-Welch Silica	2.7	3.7
Dorentruper Silica	2.7	3.7
Experiment 2		
Dorentruper Silica	2.7	3.7

[a]From Morahan et al. (*15*).

Table 10. Effect of immunopotentiators on resistance to HSV-2 infection in adult mice.

Treatment	Mortality		*p* value
	n/tot	%	
PBS	15/15	100	
C. acnes	3/15	20	< 0.05
C. parvum	4/14	29	< 0.05
Pyran	7/15	47	< 0.05

To investigate the role of interferon in host resistance to HSV, similar experiments to those performed in the EMC virus infection have been carried out. Exogenous interferon or interferon inducers have been used to enhance resistance, and anti-interferon antibody to determine if resistance is depressed. In a representative experiment shown in Table 11 mice were inoculated with HSV-2 by the IP route and then treated with either exogenous interferon or poly I:C at various times after infection. In the PBS-treated control animals, 95% of the animals died; in those treated with either interferon or the inducer, significant protection was observed when therapy was initiated as late as 48 hr post infection (*16*). In another model infection utilizing newborn mice inoculated by the intranasal route with HSV-2, treatment with poly I:C completely inhibited viral replication in lung, liver and spleen but not brain. In general, the control of HSV replication in target organs correlated with the induction of interferon in those tissues (*17*). From these and other data in the literature it is apparent that interferon or interferon inducers can protect animals against HSV infection and suggest that interferon is involved in resistance to and recovery from HSV infection. Data which more directly indicate that interferon is involved in resistance to HSV comes from the experiments of Gresser et al. (*18*), who used anti-interferon antibody to abolish

Table 11. Effect of treatment with interferon or poly I:C on mortality of mice inoculated IP with HSV type 2.

	Mortality		
Treatment	n/tot	%	p value
PBS	24/25	96	–
Interferon			
+ 1 hr	7/14	50	< 0.01
+24 hr	4/14	29	< 0.001
+48 hr	4/15	27	< 0.001
Poly I:C			
+ 1 hr	15/25	60	< 0.01
+24 hr	8/25	32	< 0.001
+48 hr	8/15	53	< 0.01

Table 12. Effect of anti-interferon globulin on HSV type 1 infection of mice.[a]

Virus dilution	Treatment	Mortality, %
10^{-2}	Normal globulin	50
	Anti-IFN globulin	100[b]
10^{-3}	Normal globulin	60
	Anti-IFN globulin	100[b]
10^{-4}	Normal globulin	0
	Anti-IFN globulin	100

[a] Modified from Gresser et al. (*18*).

[b] Mice treated with anti-interferon globulin died 3-5 days earlier than control mice

the interferon response in mice infected with HSV-1 (Table 12). In these animals the mortality rate was increased and viral replication in target organs was enhanced (*18*). These results in mice infected with HSV, also demonstrate that resistance can be altered in both directions by either increasing the amount of interferon produced or by inhibiting the endogenous response.

Murine Cytomegalovirus (MCMV) Infection of Mice

Cytomegalovirus infection of humans can result in a variety of diseases, including congenital infection of the fetus, a mononucleosis-like syndrome in patients receiving transplants or blood transfusions, a chronic infection involving numerous target organs and an asymptomatic infection of children or adults. Since the cytomegaloviruses exhibit strict species specificity, it is necessary to use closely related animal strains in experimental infections. When mice are inoculated IP with MCMV, the virus replicates to high titers in the liver, spleen, kidney and lung (*19*). This is a generalized infection involving all the major target organs and there should be maximum interaction between the viral infection and the immune system allowing one to identify numerous potential alterations of the immune response. Antibody has not been identified as a critical component of host resistance to CMV, although there is evidence that immunization may be effective in prevention of infection. The use of interferon or interferon inducers in animal or human studies have not resulted in significant protection against infection. Cytomegalovirus infections are a particular problem in the immunocompromised patient, and there are numerous studies in man and experimental animals which indicate that cell-mediated immunity is the primary component responsible for resistance to these infections. The effect of immunosuppression by cyclophosphamide, prednisolone or anti-lymphocyte serum on the immune response and mortality of mice infected with MCMV are summarized in Table 13. In immunosuppressed animals the mortality rate and reactivation of latent virus were both increased. The cell-mediated immune response to MCMV antigen, and the response to three mitogens were all suppressed. The development of humoral immunity did not appear to be altered (*20,21*). Similar data supporting the importance of cell-mediated immunity to infections by the herpesvirus group in humans have also been reported.

Evidence has been presented which suggests that experimental viral infections in animals provide ideal models for evaluating the potential immunotoxicity of substances to which humans may be exposed. It is important to note, however, that viral infections by themselves may result in alterations of immune responses in the infected host. These effects of the virus, therefore, must also be defined in any animal model utilized for toxicity studies. The MCMV infection of mice is an excellent example of how a viral infection can enhance or suppress individual components of host resistance (*22-26*). (Table 14). It appears from these data that

Table 13. Effect of immunosuppression on humoral and cellular immunity to murine cytomegalovirus infection in mice.[a]

Component tested	Result
CF antibody	Not altered
CMI to MCMV antigen	Suppressed
Mitogen response	
PHA	Suppressed
Con A	Suppressed
LPS	Suppressed
Mortality	Increased
Reactivation of latent virus	Increased

[a] Compiled from Howard et al. (*20*) and Mayo et al. (*21*).

Table 14. Alteration of host resistance by murine cytomegalovirus infection.

Component tested	Result
Antibody response, MCMV Ag	Not altered
Antibody response, other Ags	Suppressed
CMI to MCMV Ag	Not altered
Mitogen response – PHA	Suppressed
Mitogen response – Con A	Suppressed
Mitogen response – LPS	Suppressed
T-cell cytotoxicity	Not altered
NK cell cytotoxicity	Enhanced
Interferon response to MCMV	Not altered
Interferon response to other inducers	Suppressed
Macrophage function	Enhanced

[a] Compiled from data of Howard et al. (*24*), Kelsey et al. (*22*), Quinnan et al. (*23*), Stringfellow et al. (*25*) and Schleupner et al. (*26*).

Table 15. Potential effects of viral infections on the immune system.

Effect
Suppression of antibody response
Suppression of interferon production
Suppression of lymphocyte response
Nonspecific mitogen response
Specific antigen response
Suppression of macrophage function
Potentiation of macrophage function

specific responses to the virus, that is the antibody response to MCMV antigen, CMI to MCMV antigen, T-cell cytotoxicity to virus-infected cells, NK cell cytotoxicity, the interferon response to MCMV and macrophage function are either enhanced or not altered during acute MCMV infection. Certain other responses, however, such as antibody production to other antigens, lymphocyte response to mitogens, and the interferon response to other inducers are generally suppressed. This would indicate that there is specificity and regulation in the humoral and cellular response to viral infections. Immunosuppression of certain immune responses has also been documented in other viral infections such as influenza and measles (Table 15). In determining the effect of environmental substances on the immune system during a model viral infection, the components of host resistance for evaluation will have to be selected carefully such that an effect induced by a foreign substance can be distinguished from those caused by the viral infection.

Experimental viral infections in animals appear to be ideal models for assessing changes in the immune system. The virus-host interaction, however, is extremely complex, involving numerous components of host resistance. In any particular viral infection there is no one component responsible for resistance, instead there are multiple interactions among the various components. This concept is supported by the relationship of macrophages and B-cells in the production of antibody, the activation of macrophages by interferon, and the regulatory activity of helper and suppressor cells and lymphokines. One of the limitations in the use of these models is that the viral infection may also alter host responses. It should be stressed that the animal model cannot be utilized effectively by just treating the animal with a particular substance, infecting with virus and then haphazardly determining effects on components of host resistance. To provide optimal information it is important that one first understand the pathogenesis of that particular viral infection being used and the specific and nonspecific host responses to that infection.

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Environmental Health Perspectives
Vol. 43, pp. 81-88, 1982

Application of Tumor, Bacterial and Parasite Susceptibility Assays to Study Immune Alterations Induced by Environmental Chemicals

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Model systems to study the effects of chemicals of environmental concern on bacterial and parasitic diseases as well as the immunosurveillance and destruction of transplantable tumor cells were described and evaluated. Studies were conducted in female B6C3F1 mice following adult or pre/postnatal exposure to several prototype chemicals. The prototype chemicals employed included the synthetic estrogen diethylstilbestrol (DES), the polycyclic aromatic hydrocarbon benzo(a)pyrene (B[a]P), and the carcinogenesis promoting agent 12-0-tetradecanoylphorbol-13-0-acetate (TPA).

The host resistance models employed depend primarily on functional thymus-dependent immunity, although humoral immunity is suggested to have a role in the parasite model as well. These models include: subcutaneous challenge with a dose of PYB6 tumor cell causing a 10-20% incidence (TD_{10-20}) of tumor; intravenous challenge with B16 melanoma cells; challenge with a dose of ***Listeria monocytogenes*** causing a 10-20% incidence of mortality (LD_{10-20}); challenge with a dose of *E. coli* lipopolysaccharide endotoxin causing a 10-20% incidence of lethality (LD_{10-20}); and challenge with larvae of ***Trichinella spiralis*** for parasite expulsion kinetic studies.

Increased mortality was observed following ***Listeria monocytogenes*** challenge in DES-exposed mice. B(a)P and TPA exposure did not alter host resistance to this organism. The increased mortality observed following DES was associated with a significant increase in the number of viable Listeria in the spleens and livers at 4 days, a time when T-cell immunity is thought to be expressed, but bacterial counts were similar to control mice at day 1, a time when $M\Phi$ are thought to exert their greatest effect. These data suggest that the increased Listeria susceptibility found following DES exposure may result from a T-cell defect, although the intracellular killing capacity of DES-treated $M\phi$'s has not been well examined.

Tumor susceptibility studies following challenge with 5×10^3 viable syngeneic PYB6 tumor cells revealed that nontreated adult B6C3F1 mice resisted tumor formation, with only a 10-20% incidence of tumor formation. In contrast, mice exposed to DES or TPA as adults had a tumor frequency of from 70-100% following TPA and up to 90% following DES exposure. In all cases the tumors were progressive and resulted in death. B(a)P did not alter the frequency of tumor incidence from controls in this model.

Preliminary data, using the B16 melanoma intravenous challenge model and ^{125}IUdR to quantitate tumor mass revealed this model was sensitive to non-specifically activated macrophage kill. DES treated mice with activated macrophages did not demonstrate increased tumor mass, while mice exposed to TPA or the potent immunosuppressive agent cyclophosphamide had a significantly increased tumor mass in their lungs.

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Expulsion of *Trichinella spiralis* adults from the gut also apparently required functional T-cells and possibly some element of humoral immunity. Mice exposed to DES and B(a)P exhibited increased numbers of adult worms in the gut at day 14.

Sensitivity to gram-negative endotoxin (LPS) was apparently increased following exposure to DES or B(a)P. These data suggest that the detoxification of LPS is related to an intact MΦ population. The data presented here demonstrate the sensitivity of the host resistance assay panel proposed for detecting immune alteration. Alteration of T-cell function appeared to correlate with increased susceptibility to bacterial and tumor cell challenge.

Introduction

During the past few years there has been a renaissance of interest in the development of sensitive and reproducible rodent host resistance models to define immunological dysfunction following exposure to drugs or chemicals of environmental concern. These assays are needed to improve the data base for the safety assessment of these agents and to provide better correlates with the numerous *in vitro* assays of immunological function. This interest in providing better correlation and interpretation between an alteration in one or several measurable immunological parameters and host resistance effects, stems from the well known association between congenital immunodeficiency syndromes and an increased frequency of neoplasia (*1*, *2*) or infectious diseases. In addition, renal transplant patients maintained on chronic therapy with hydrocortisone or immunosuppressive agents to sustain an allograft have been shown to have a much higher (2.5 ×) frequency of solid tumors and an increased frequency of infectious disease (*3*, *4*). Several groups (*5*, *6*) have observed a correlation between depressed delayed cutaneous hypersensitivity responses (DHR) to recall antigens (i.e., partial or complete anergy) and an increased frequency of bacterial sepsis and mortality following major surgery. Collectively these clinical and epidemiological data support a strong association between immune dysfunction and altered host resistance.

Altered immunological function, as indicated by the inability to be sensitized for DHR to recall antigens and increased susceptibility to certain infectious agents have likewise been observed in congenital athymic nude (nu/nu) mice (*7*, *8*) and rats (rnu/rnu) (*9*). Likewise, Law et al. (*10*) observed that neonatal thymectomy increased the susceptibility of mice to polyoma virus induced tumors. The application of immunologic and host resistance assays to study rodents following exposure to chemicals has indicated that certain chemicals can result in immune dysfunction (*11-13*). In addition, such exposure often leads to altered host resistance to bacteria (*14*, *15*), viruses (*16-18*), parasites (*19*, *20*), or transplantable syngenic tumor cells (*20-22*) as well as spontaneous tumors (*23*, *24*).

Although there is general agreement that severely depressed cell-mediated immunity (CMI) or humoral-mediated immunity (HMI) results in altered host susceptibility to infectious agents, there is no general agreement regarding the effects of subtle or chronic immunosuppression nor is there agreement about which measurable immunological function(s) most consistently predicts altered host resistance. The recent emphasis on evaluating chemical induced immunotoxicity has resulted in a concerted effort to optimize the sensitivity and reproducibility of these models, to re-examine the application of these models and to improve the data base regarding their correlation with other immune function parameters which are readily measured *in vitro*. These pursuits have been a major goal of our laboratory and will be described here.

Operative Immunological Mechanisms for Resistance to Bacterial Infections and Neoplasia

Bacterial infections are classified as either acute, chronic or toxigenic. The former is illustrated by Staphylococcus or Streptococcus infections in which non-specific phagocytic mechanisms and later specific antibody mediated mechanisms are primarily operative (Fig. 1). Antibodies serves to enhance the phagocytosis and killing of pathogenic microorganisms by polymorphonuclear leukocytes (PMN) and macrophages (MΦ) through opsonization or neutralization of specific bacterial toxins by preventing binding of the toxin to their specific receptors. Chronic infections, caused by organisms such as *Listeria monocytogenes* or *Mycobacterium tuberculosis*, that are facilitative intracellular pathogens multiply within the phagocytic cell and thus prevent PMN killing. CMI enhances the killing efficiency of the macrophage in these infections through lymphokines such as macrophage chemotactic factor (CF), macrophage migration inhibition factors (MIF) and macrophage activation factor (MAF). Ultimately, CMI plays the primary role in the control and eradication of these often persistent and chronic agents (Fig. 1). Toxigenic infections result from the production of toxins by certain

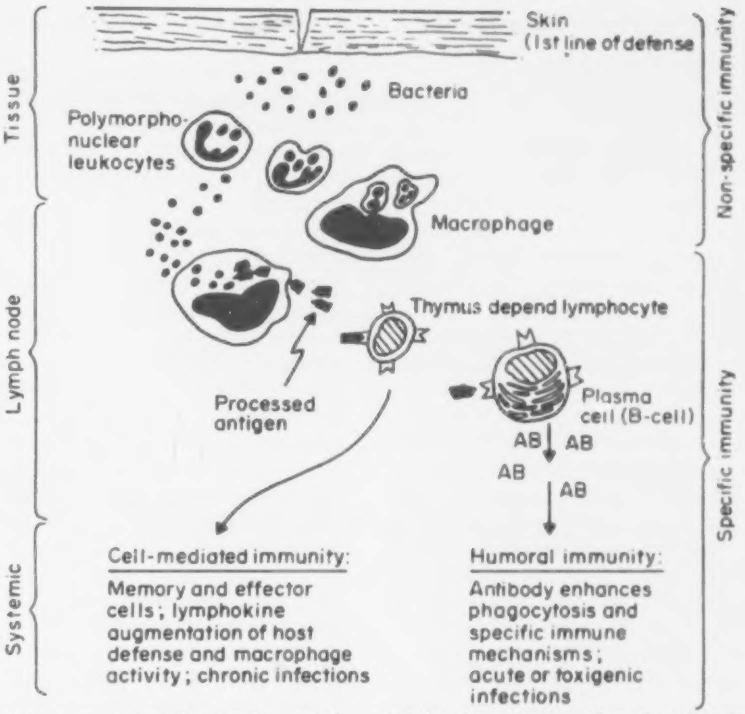
Skin
(1st line of defense
Bacteria
Polymorphonuclear leukocytes
Macrophage
Thymus depend lymphocyte
Processed antigen
Plasma cell (B-cell)
AB
AB
AB
AB
Cell-mediated immunity:
Memory and effector cells; lymphokine augmentation of host defense and macrophage activity; chronic infections
Humoral immunity:
Antibody enhances phagocytosis and specific immune mechanisms; acute or toxigenic infections
Tissue
Lymph node
Systemic
Non-specific immunity
Specific immunity

FIGURE 1. Diagrammatic representation of host resistance to bacteria indicating roles played by cell-mediated and humoral immunity.

bacteria and require the production of specific antibody for their neutralization (e.g., tetanus toxin) with little if any role played by CMI.

An imbalance or transient dysfunction of the immune surveillance mechanisms is believed to facilitate the development of neoplastic disease (*25*). For example, a newly transformed or transplanted tumor cell may present a foreign antigenic configuration to the host who in turn develops an immunological response designed to eradicate the tumor cell. This response is believed to be similar to that mounted against an allograft and its relatively weak antigens associated with the tumor cell membrane is predominantly T-lymphocyte-dependent, involves a cascade of cell types and events collectively known as cell-mediated immunity and culminates in the production of T-effector cells and activated macrophages (*26*).

Chemicals, Exposures and Assay Methods

We have investigated several chemicals of environmental concern by short term exposure in adult female B6C3F1 mice. Diethylstilbestrol (DES) was obtained from Sigma Chemical Company (St. Louis, Mo). Female B6C3F1 mice, 6 to 8 weeks old, exclusive of the control group were injected subcutaneously (sc) with 0.2, 2 or 8 mg/kg body weight of DES dissolved in 0.1 ml of corn oil for five consecutive days. Benzo(a)pyrene [B(a)P] was kindly supplied by Dr. Douglas Walters through the NTP chemical repository and administered in corn oil by sc injections. Adult female B6C3F1 mice received a total dose of 50, 200 or 400 mg/kg body weight of B(a)P over a 14-day period exclusive of controls. The phorbol ester, TPA (12-0-tetradecanoylphorbol-13-0-acetate) was obtained from Chemicals for Cancer Research (Eden Prairie, MN), dissolved in corn oil and administered by sc injections. Adult female B6C3F1 mice received a total dose of 40, 400 or 800 μg of TPA over a 14 day period exclusive of controls. Control mice for the dosage groups described above received corn oil (0.1 ml) on the same schedule as chemically treated groups in each case. Immunological and host resistance studies were performed 2-5 days following the last dose.

The battery of assays employed for host resistance evaluation has been previously described in detail by Dean and co-workers (*13*, *21*, *27*).

Altered Resistance to Tumor Cell Challenge Following Chemical Exposure

In normal mice most transplantable syngeneic tumors can be titrated to a cell concentration that will produce tumors in 100% (TD_{100}), 50% (TD_{50}) or 10% (TD_{10}) of the challenged mice. Treatment of mice with known immunosuppressive agents which alter thymus-dependent lymphocyte numbers or function prior to tumor cell challenge will result in a significantly higher frequency of tumors (*21*). Conversely, agents which stimulate immunologic function will facilitate resistance to tumor development in mice given a TD_{80-100} dose of tumor cells. These same agents will likewise facilitate or diminish the frequency or latency (i.e., time to tumor development) of spontaneous tumor in high risk mouse strains. Table 1 summarizes the effect of DES, B(a)P and TPA exposure in adult female B6C3F1 mice on the incidence of tumors following a TD_{10-20} challenge dose of 5×10^3 viable PYB6 tumor cells. A significantly increased frequency ($p < 0.05$) of tumors was observed in mice exposed to the medium (80%) and high (90%) dose of DES and all doses of TPA (70-100%). B(a)P exposure did not modulate the out-growth or development of this tumor. These results are consistent with the immunological alterations reported in the companion presentation (*28*) which indicated that DES and TPA effect predominantly T-cell mediated immune function while B(a)P exposure alters primarily the HMI response.

Table 1. Effect of chemical exposure on tumor frequency following challenge with PYB6 tumor cells.

Chemical treatment	Total dose	No. tumors[a] No. mice challenged	Tumor frequency, %
DES[b]	0	2/10	20
	1.0 mg/kg	3/10	30
	10.0 mg/kg	8/10[c]	80
	40.0 mg/kg	9/10[c]	90
B(a)p[d]	0	2/10	20
	50 mg/kg	2/10	20
	200 mg/kg	2/10	20
	400 mg/kg	1/10	10
	800 mg/kg	2/10	20
TPA[e]	0	4/20	20
	40 μg/mouse	8/10[c]	80
	400 μg/mouse	7/10[c]	70
	800 μg/mouse	8/8[c]	100

[a] Mice were challenged subcutaneously with 5×10^3 syngeneic PYB6 cells 3 days following the last exposure and palpated twice weekly for 60 days.

[b] Mice were treated with 0.2, 2 and 8 mg/kg diethylstilbestrol (DES) (Sigma Chemical Co.) in corn oil daily for 5 days and assays were performed on days 7-10.

[c] The tumor frequency was significantly different from corn oil treated controls by chi-square analysis at $p < 0.05$.

[d] Mice were given 5, 20, 40 and 80 mg/kg of benzo(a)pyrene (B(a)P) in corn oil by gavage for 10 treatments over a 14 day period.

[e] Mice were injected subcutaneously with 10, 100 and 200 μg of the phorbol ester TPA (12-*O*-tetradecanoyl-phorbol-13-*O*-acetate) dissolved in corn oil twice weekly for 2 weeks.

Previous studies (*29*) in both male and female C3H mice demonstrated a significant increased dose-related incidence of spontaneous mammary tumors following DES exposure. They likewise (*29*) demonstrated a requirement for murine mammary tumor virus (MMTV) for the mammary tumor inductive effects of DES. The incidence of spontaneous mammary tumors following DES was additionally enhanced by radiation exposure, another agent which alters T-cell function (*30*). In addition, Bern et al. (*31*) found that neonatal exposure to estrogens likewise gave an increased incidence and earlier appearance of mammary tumors. McMartin et al. (*32*) has suggested that the effect of DES on mammary tumor incidence is via the stimulation of pituitary and serum prolactin which in turn stimulates MMTV production. However, it is possible that impairment of T-cell immunocompetence or Mϕ function is a major cofactor in the enhancement of mammary tumor frequency following DES exposure and accounts for the increased tumor frequency we have observed following DES exposure (*29*). This speculation is supported by the knowledge that a potent immunosuppressive drug such as cyclophosphamide enhances the frequency of mammary tumors in low MMTV expressor strains (BALB/c-CRGL) (Lopez, personal communication).

B(a)P failed to alter susceptibility of B6C3F1 mice to challenge with PYB6 tumor cells. This was consistent with the observation reported in this volume (*28*) and previously (*33-35*) that B(a)P primarily affects B-cell response. Immunity to virus and chemically induced tumors is primarily mediated by cell-mediated and not humoral immunity (*10, 36*). The previous reports of an association between depressed humoral antibody responses and increased tumor frequency in B(a)P-treated mice (*34*) probably represent separate nonassociated events based on the minimal alterations in immunological and host resistance parameters observed in our comprehensive study.

Finally, TPA induced a statistically increased frequency in the outgrowth of PYB6 tumors following challenge. This is consistent with preliminary data which indicates that the concentration of TPA that altered tumor outgrowth likewise effects T-cell mediated immunity.

In a search for a more rapid and quantitative

Table 2. Effect of chemical exposure on the growth of B16 melanoma in the lung following intravenous challenge.

B 16 melanoma challenge	Chemical treatment	Colonies/lung	$\bar{X}$ cpm of 3H-TdR incorporation ± SEM[a]	Change in ^{3}H-TdR over control, %[b]
–	None	0	239 ± 15	—
+	Corn Oil	8	465 ± 138	+95
+	CY (180 mg/kg)	>50	6359 ± 873[c]	+2550
+	DES (10 mg/kg)	0	296 ± 32	+23
+	TPA (400 μg/mouse)	>50	1808 ± 429[c]	+653

[a] Six mice per group were injected with 5×10^4 B16F10 melanoma cells by the intravenous route 3-5 days following the last exposure (day 0). All mice received 1 mg of FUDR on day 20 following at 1 hr by approximately 1×10^6 CPM of ^{125}IUdR (IP) and were sacrificed 18-20 hr later. The lungs were perfused with saline, placed in a counting tube and counted for 10 min in a Packard Biogamma Counter. The data are expressed as mean counts per minute ($\bar{x}$ CPM) of ^{3}H-TdR incorporation ± standard error of the mean (SEM) from six mice/group.

[b]
$$\%\ \text{Change} = \frac{\bar{x}\ \text{(cpm) of } ^{125}\text{IUDR in the lungs of non-tumored mice}}{\bar{x}\ \text{(cpm) of } ^{125}\text{IUDR in the lungs of treated and tumored mice}} - 1 \times 100$$

[c] Significantly different from mean CPM in control mice at $p < 0.01$ by Student's *t*-test.

method of assessing tumor resistance we have used an ^{125}IUdR (^{125}I-iododeoxyuridine) pulsing technique (*27*) to quantitate tumor mass in the lungs of chemical treated mice at 21 days following an intravenous challenge with the B16F10 melanoma line of Fidler (*37*). In this assay mice are challenged with 5×10^4 viable tumor cells at 2-3 days following their last dose of chemical. This model assumes that normal surveillance and immunological mechanisms operating systemically in the lungs of mice resist or destroy a large portion of the tumor cell inoculum. The remaining tumor cells which escape surveillance primarily develop foci in the lungs, appearing as a black mass of tumor which can easily be enumerated. Treated and tumored mice are then injected with approximately 1×10^6 cpm of ^{125}IUdR on day 20 following tumor cell injection and the lungs are removed on day 21, perfused with saline, and the amount of ^{125}IUdR incorporation determined by a gamma counter.

Table 2 summarizes preliminary studies of the lung tumor mass found in B6C3F1 mice following exposure to the standard immunosuppressive agent cyclophosphamide (CY, 180 mg/kg) and the chemicals DES (10 mg/kg) and TPA (200 μg) followed by challenge with B16F10 melanoma cells. As expected, an immunosuppressive dose of CY resulted in a 2500-fold increase in tumor mass as estimated by ^{125}IUdR and TPA exposure resulted in a 653-fold increase in tumor mass. These data roughly correlated with the number of colonies seen on the surface of the lungs (i.e., 8 versus > 50). Corn oil (i.e., carrier) or DES did not significantly increase lung tumor mass, in fact DES treated mice had a lower incorporation rate than controls. This apparent paradox produced by DES in this model may be due to the exquisite sensitivity of the B16F10 tumor line to nonspecifically activated macrophage cytotoxicity (*37*) and the potency of DES as a Mϕ activator (*38*, *39*). Morahan et al. (*40*) have previously shown that low MW pyran copolymers will potentiate the tumorcidal activity of macrophages against the transplantable form of B16 melanoma. Our interpretation of increased susceptibility to B16F10 tumor cells in CY and TPA treated mice is consistent with the adverse effect of both CY (*28*) and TPA (*41*) on T-cell mediated immunity. B(a)P has not yet been examined in this model. This model may also be a useful procedure for screening chemicals suspected of immunopotentiatory effects.

Altered Susceptibility to Listeria Infections Following Chemical Exposure

The role of immunocompetent T-cells and macrophages in controlling the intracellular replication and destruction of the gram positive bacteria *Listeria monocytogenes* has been well documented (*42*, *43*). Recently, Newborg and North (*44*) re-examined this model using nude mice and found that survival during listeriosis ultimately requires the generation of T-cell mediated immunity. In our studies, control and chemical treated mice were challenged with 1×10^6 viable Listeria which produced a mortality frequency of approximately 10-20% ($LD_{10\text{-}20}$) in normal B6C3F1 mice. Table 3 summarizes mortality following Listeria challenge in mice exposed to DES, B(a)P and TPA. DES significantly reduced resistance to *Listeria* at all doses of chemicals (i.e., 100% mortality). The mean latency to death was likewise reduced. In contrast, B(a)P and TPA did not significantly alter Listeria resistance.

The increased mortality we observed following DES was associated with a significant increase in the number of viable Listeria in the spleens and livers at 4 days (*20*), a time when T-cell immunity is

Table 3. Effect of chemical exposure on mortality to *Listeria monocytogenes* challenge.[a]

Chemical	Dose	No. deaths No. inoculated	Mortality, %
DES	0	2/10	20
	1.0 mg/kg	10/10[b]	100
	10.0 mg/kg	10/10[b]	100
	40.0 mg/kg	10/10[b]	100
B(a)P	0	0/10	0
	50 mg/kg	1/10	10
	200 mg/kg	0/10	0
	400 mg/kg	2/10	20
TPA	0	4/23	17
	40 μg	0/10	0
	400 μg	0/10	0
	800 μg	0/10	0

[a] Animals were challenged with 1 × 10^6 viable bacteria and mortality followed in 14 days.

[b] Significantly different from control mice by chi-square analysis.

thought to be expressed. Bacterial numbers similar to control mice were observed at day 1 post-infection, a time when Mϕ are thought to exert their greatest effect. In a previous study, Heller (*45*) found that synthetic estrogens increased phagocytosis, but that enhanced phagocytosis did not result in enhanced protection against microbial infection. Thus, the altered resistance to *Listeria* observed appears to be related to the functional T-cell defect observed following DES and reported in the companion presentation (*46*). B(a)P was not expected to alter resistance to *Listeria* since antibodies have no defined role in controlling listerosis (*44*) and B(a)P primarily effects B-cell function (*34, 41*). In contrast, the lack of effect of TPA on Listeria resistance was unexpected since in preliminary studies TPA altered T-cell function and increased the susceptibility to PYB6 tumor cells. Thus, the T-cell dysfunction observed following TPA appears to be unique to tumor resistance. This observation awaits confirmation and definition through further studies.

Altered Hypersensitivity to Endotoxin Following Exposure

The detoxification of gram negative bacterial endotoxin is believed accomplished primarily by liver macrophages (*47*) and endotoxin challenge is thought to mimic the response to gram-negative bacteria. Increased sensitivity to endotoxin has been observed following the administration of known reticuloendothelial system stimulants such as glucan and zymozan or certain environmental chemicals (*11*). In the studies described here, B6C3F1 mice were challenged intravenously with 650 μg of *Escherichia coli* endotoxin 2-3 days following the last chemical dose and animal survival was observed for 48 hours. Table 4 summarizes mortality at 48 hr following challenge with endotoxin in mice exposed to DES, B(a)P and TPA. Mortality was significantly increased (44-90%) in DES treated mice. B(a)P treated mice likewise had increased mortality following endotoxin challenge (30-80%). TPA treated mice did not have increased hypersensitivity to endotoxin. The pattern of increased endotoxin sensitivity following chemical treatment appears to remain ill-defined correlating with Mϕ alterations in the case of DES. Significantly increased mortality to endotoxin following B(a)P exposure only at the high dose may reflect macrophage toxicity. Thus, hypersensitivity to endotoxin appears to offer a sensitivity indicator of chemical immunotoxicity.

Table 4. Effects of chemical exposure on endotoxin hypersensitivity.

Chemical	Mortality, %[a] Control	Low	Medium	High
DES	0	67[b]	90[b]	44
B(a)P	0	30	40	80[b]
TPA	0	10	0	10

[a] After 48 hr in mice challenged with 650 μg of *E. coli* endotoxin.

[b] Significantly different from control by chi-square analysis at $p < 0.05$.

Altered Expulsion of Adult Trichinella Following Chemical Exposure

The role of T-cell mediated immunity in the expulsion of adult *Trichinella spiralis* has been clearly shown by Larsh and associates (*48*). In this model mice are challenged with 200 larvae following chemical exposure and are then sacrificed on day 14 and the number of adult worms in the gut are enumerated. Table 5 summarizes adult worm counts following exposure to DES, B(a)P and TPA in B6C3F1 mice. DES treated mice had a significantly greater number of adult worms ($p < 0.01$) in their gut at day 14 than did control mice. These data demonstrate that DES treatment of naive mice prior to larvae challenge reduced the normal expulsion of adult worms (63%) and substantiated the role of T-cell immunocompetence in eradicating the host of adult worms. Similarly, nude mice have been shown to have reduced expulsion rates with adult worms lingering for up to 83 days (*49*). B(a)P

Table 5. Effect of chemical exposure on *Trichinella spiralis* expulsion.

Chemical treatment	Dose, mg/kg	Adults recovered[a]	% of control,[b]
DES	0	1.1 ± 0.8	0.7
	1.0	0.9 ± 0.5	0.6
	40	96.0 ± 14.7[c]	62.8
B(a)P	0	1.3 ± 0.7	1.4
	50	3.0 ± 1.0	3.3
	200	5.0 ± 0.7[d]	6.4
	400	49.8 ± 12.6[c]	54.2
TPA	0	4.8 ± 2.5	4.3
	40	7.5 ± 3.5	6.7
	400	1.5 ± 1.1	1.3
	800	5.8 ± 3.4	5.2

[a] Average adult counts ± SEM of 7 mice/group examined at 14 days post infection.

[b] % Control = % of adult worm remaining at day 14 versus controls killed on day 7.

[c] Significantly different by Student's *t*-test at $p < 0.001$.

[d] Significantly different by Student's *t*-test at $p < 0.01$.

also reduced parasite expulsion (54%), at the two highest doses administered. This finding may suggest that humoral immunocompetence is also required for efficient expulsion. Finally, TPA had no effect on adult worm expulsion.

Conclusion

In conclusion, the studies described here demonstrate that adult exposure of female B6C3F1 mice to various chemicals can severely impair host resistance to syngeneic tumor cells, Listeria, endotoxin and *T. spiralis* challenge. These studies also point out the sensitivity of and significance of examining host resistance parameters when evaluating the immunotoxicity of environmental chemicals or drugs. The study of chemical toxicity might also provide a powerful tool for dissecting mechanisms of altered host resistance and immunocompetence. The data presented in this and the companion report (*28*) strongly suggest that exposure of adult mice to DES, B(a)P or TPA can result in thymus atrophy, impaired DHR responses, impaired T-cell responses *in vitro*, impaired AB production, and enhanced *in vitro* peritoneal macrophage activity as measured by phagocytosis and cytostasis of tumor target cells. The host resistance models described are strongly dependent on depressed T-lymphocyte immunocompetence if effects are to be observed, although a secondary specific defect in peritoneal $M\phi$ function (i.e., associated with DES) cannot be ruled out and will require further clarification through more specific *in vitro* examination of the intracellular killing capacity of macrophages from exposed mice. Finally, these assays appear quite sensitive for detecting chemical induced immune dysfunction.

The authors greatly appreciate the assistance of Ms. Beth deBrito in the preparation of this manuscript.

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Environmental Health Perspectives
Vol. 43, pp. 89-97, 1982

Macrophage Induction of T-Suppressor Cells in Pesticide-Exposed and Protozoan-Infected Mice

by Leland D. Loose*

The use of infectious pathogens has allowed the detection of the development of synergism between pathogens and ubiquitous environmental chemical contaminants. This synergism has been demonstrated to result in a state of immunosuppression which either did not occur in the independent and singular presence of the chemical or pathogen and/or was greater than additive when both were combined. The immunosuppression was distinct with regard to the organochloride used and, therefore, is not a ubiquitous characteristic of all organohalides. The production of a macrophage soluble factor which appeared to induce T-suppressor cells was demonstrated in hepatic Kupffer cells from mice administered 5 ppm of dieldrin for 10 weeks and then infected with *Leishmania tropica* promastigotes. The factor was not generated in mice administered dieldrin and infected with malaria nor in mice administered only dieldrin nor in mice only infected with *Leishmania*. Additional studies revealed a profound impairment in macrophage antigen processing with macrophages obtained from mice administered dieldrin. The use of pathogen models may allow the immunosuppressive potential of environmental chemical contaminants to be expressed in a more sensitive manner.

Introduction

Previous studies have demonstrated that environmental chemical contaminants impair host defense responses to a malaria challenge and to gram-negative endotoxin (*1*). In conjunction with these alterations, a significant reduction in antibody formation to sheep erythrocytes, (SRBC), a T-dependent and macrophage-processed antigen, was observed (*2*). These functional alterations were demonstrated in the absence of any lymphoid histopathologic changes. Further studies to examine cell-mediated immunity (CMI) in the xenobiotic treated mice revealed an essentially normal CMI responsiveness as measured by the graft-versus-host assay, mixed lymphocyte culture reaction, polyclonal mitogen activity and lymphocytotoxicity response (*3*). Since it was felt that the CMI parameters which were measured and which were unaltered were, in general, macrophage-independent whereas the response to malaria, endotoxin and SRBC were macrophage-dependent, the cellular lesion responsible for the impaired malaria, endotoxin and SRBC responses could reside at the level of the macrophage.

Measurements of macrophage affector activity, i.e., cellular respiration, phagocytic activity, phagocytic capacity and chemotaxis revealed no alteration in these parameters (*4*). However, macrophage effector activity, i.e., antigen processing and tumor cell killing were significantly impaired (*5*). Therefore, it was speculated that if the cellular lesion responsible for the xenobiotic-induced immunosuppression was at the level of the macrophage, that the lesion could be due to: a functional impairment in the macrophage, induction of a population of suppressor macrophages, production of suppressor

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factors from the macrophage or a combination of one of the above.

Therefore, further studies were conducted to determine the contribution of the macrophage to xenobiotics-induced immunosuppression. The possible development of a synergistic reaction between the influence of the xenobiotics on the immune response and a concomitant protozoan infection was ascertained concurrently.

Materials and Methods

Animals

Male BALB/c mice (18-20 g) were used throughout all studies.

Chemicals

Dieldrin (Shell) and hexachlorobenzene (HCB, Eastman) were administered in the diet at 1 and 5 ppm and 5 and 100 ppm, respectively. Control mice received untreated powdered Wayne Lab Blox. Food and water were provided *ad libitum*, and a 12:12 day:night photoperiod was maintained. Dietary administration of the test chemicals was for 3, 5 or 10 week periods.

Parasites

Plasmodium berghei (NYU-2) was passaged weekly by intraperitoneal (IP) injection of 1 × 10^6 infected erythrocytes. The survival time of infected mice injected with this strain of murine malaria at the stated inoculum was approximately 10-12 days. *Leishmania tropica* was initially derived from a biphasic blood agar medium and then adapted to a defined culture medium consisting of MEM 199 with 10% heat inactivated fetal calf serum. Subcultures were made weekly. The parasite stage used was a promastigote in a mid- to late-log phase. Survival time of BALB/c mice injected intradermally (ID) with 1 × 10^6 promastigotes was approximately 15-20 weeks.

Antibody Formation

Antibody production was assessed using the direct Jerne and Nordin (*6*) plaque assay as previously described (*2*). Plaque formation to polyvinylpyrrolidine (PVP, Sigma), a T-independent antigen, utilized passively sensitized SRBC coated with PVP by the method of Boyden (*7*). Preliminary studies revealed that the kinetics of splenic plaque formation in experimental and control mice were comparable and, therefore, only day 4 was used to subsequently enumerate plaques.

Mononuclear Spleen Cell Populations

Splenic T- and B-lymphocytes were enumerated as previously described (*8*). T-cells were detected by the presence of theta antigen (Thy-1) and subsequent cytotoxicity induced by anti-theta serum. B-cells were detected by the presence of surface immunoglobulin (Ig) using fluorescein-tagged, goat anti-mouse Ig. Splenic macrophages were stained by the method of Yam, Li and Crosby ([illegible]), which detects the presence of surface neutral esterase, a cytochemical marker for macrophages.

Lymphocyte Blastogenesis

Spleens were removed aseptically from mice, controls and experimentals, at the stated intervals (see Tables 5-8), and teased apart in cold RPMI 1640 and strained through a 10xx silk cloth. This single cell suspension was then passaged through nylon wool (Leuko-Pak leukocyte filter, Fenwal Labs, Deerfield, Ill.) which was soaked in saline for 2 hr at 37°C then rinsed three times in doubly distilled water for 3 days, autoclaved and then dried. A 60-ml disposable syringe barrel was then packed with 6 g of nylon wool and then washed with 20 ml of RPMI 1640 containing 5% fetal calf serum. The columns were then drained and placed in a 37°C incubator for 1 hr prior to being loaded with 10^8 spleen cells in 20 ml of RPMI 1640. The column, with the cells, was incubated at 37°C for 45 min prior to eluting the cells at a 1 ml/min rate. The T-cell enriched suspension was then concentrated by centrifugation at 250 g for 15 min. Cell viability, as determined by trypan blue dye exclusion, was > 85%, and > 95% of the cells were theta positive. The enriched T-cell population was then dispensed in 200 μl volumes containing 4 × 10^5 cells into Linbro flat-bottomed microtest plates (Flow Lab). The RPMI 1640 medium was supplemented with 2mM L-glutamine, 10% fetal calf serum 50-100 units of penicillin and 50-100 μg of streptomycin/ml. In addition, phytohemagglutinin (PHA-M, grade, Difco) was added at a concentration of 0.1 μg/well. The plates were incubated at 37°C in humidified 5% CO_2 for 48 hr, after which 0.1 μCi of ^{3}H specific activity 5 Ci/mmole) was added and the plates incubated for an additional 16 hr prior to harvest. Cell harvesting was conducted as previously described (*3*), and data are presented as cpm × 1[illegible]. Anti-theta serum treatment of the nylon wool enriched T-cell preparation utilized a 1:80 dilution of anti-Thy-1 antiserum (Cedarlane Labs) and a 1:10 dilution of

Low-Tox abbit complement incubated together for 30 min at 7°C. Mitomycin C treatment consisted of incubati the nylon wool passaged cells (10^7/ml) with 20 of mitomycin C (Sigma) for 25 min at 37°C. Th cells were washed three times in RPMI 1640 pri to use in blastogenesis studies (*3*).

Macro age Assay

Alveol splenic and peritoneal macrophages were isolated previously described (*4*). Briefly, alveolar macr hages were isolated by lavage of the lung with sali , peritoneal macrophages were isolated by saline avage of the peritoneal cavity, and splenic macr hages were isolated by surface adsorption. He tic Kupffer cells were isolated by partial enzymat digestion of the liver as described by Stege et al. (*10*). The macrophage preparations were incubated overnight in RPMI 1640 plus 10% FCS in a 5% CO_2:humidified atmosphere at a cell concentration of 1×10^7 cells/culture plate. The following day the cell supernatant was decanted off and centrifuged at 250 g for 20 min to remove cell debris. A 50 µl aliquot of this supernatant was then added to the T-cell blastogenesis cultures, replacing 50 µl of the RPMI 1640 medium. PHA-induced blastogenesis studies were then conducted as described above.

Macrophage antigen processing of SRBC, a T-dependent antigen which requires macrophage cooperation for immunogenicity, was conducted as previously described (*5*).

Statistics

All data are reported as the mean with significance denoted by an asterisk where $p < 0.05$. Significance was det mined by using the Student's *t* test. Significa ce for mortality data was calculated using the time ffect evaluation of Litchfield (*11*).

Results

As can be seen in Table 1, the addition of either 1 or 5 ppm of dieldrin to the diet of mice exacerbated the lethality induced by a challenge infection with either the malaria parasite *Plasmodium berghei* (NYU-2) or *Leishmania tropica*. The lethality synergized by dieldrin and the parasites appeared to be both time and dose related. However, mice administered 5 or 100 ppm of HCB and then challenged with malaria or Leishmania expressed a synergy only with Leishmania and not malaria. The mortality induced by Leishmania was more marked in dieldrin-exposed mice than in HCB-exposed mice.

When the dieldrin and HCB-treated mice which were infected with malaria or Leishmania and then evaluated for their ability to produce antibody to SRBC, a suppressive synergism was noted primarily in HCB-treated mice (Table 2). In these mice, the splenic plaque forming response was more suppressed in the combination of HCB + infected mice than in those only HCB-treated or only infected. In dieldrin-treated mice, a synergism was observed only in mice which received 5 ppm of dieldrin plus infection with Leishmania.

Although a suppressed SRBC response was noted in HCB-treated mice infected with either malaria or Leishmania, a decreased antibody formation to PVP, a T-independent antigen, was observed only in Leishmania-infected mice exposed to dieldrin or HCB. Malaria did not act synergistically with dieldrin or HCB to elicit an impaired response to PVP (Table 3). The decreased antibody formation to PVP in dieldrin- and HCB-treated mice infected with Leishmania was time- and dose-related in dieldrin-treated mice but was only time-related in HCB-treated mice.

An examination of the number of splenic T-cells, B-cells and macrophages revealed no consistent significant alterations in the cell types in either

Tal 1. **Mortality in *Plasmodium berghei* and *Leishmania tropica* infected mice administered dieldrin or HCB.**[a]

	Mean survival time, days[b]							
	Dieldrin, 1 ppm		Dieldrin, 5 ppm		HCB, 5 ppm		HCB, 100 ppm	
Weeks	Malaria	Leishmania	Malaria	Leishmania	Malaria	Leishmania	Malaria	Leishmania
3	10.4	123.5	10.8	80.8*	12.0	97.6*	11.1	73.7*
6	10.6	81.2*	7.2*	57.7*	11.9	83.1*	9.8	60.0*
10	7.1*	53.9*	4.7*	23.0*	10.8	75.6*	7.0*	41.1*
Control	12.3	119.0						

[a] Male LB/c mice (18-20 g) were administered dieldrin or HCB at 1 and 5 or 5 and 100 ppm, respectively, for 3, 6 or 10 weeks and then chal ged with either 1×10^5 *Plasmodium berghei* (NYU-2) infected erythrocytes (IP) or 1×10^6 *Leishmania tropica* promasti es injected ID.

[b] Mean vival time (days) is denoted for experimental and control mice and significance is indicated by an asterisk where $p < 0.05$; $n = 12$ p group. Following injection of the parasite(s) all dietary administration of HCB or dieldrin ceased.

Table 2. Direct splenic plaque formation to SRBC in mice infected with malaria or Leishmania with administration of dieldrin or HCB.[a]

	Chemical treatment		Splenic PFC/10^6 cells		
Time, weeks	Agent	Concn, ppm	No infecting organism	Malaria	Leishmania
–	None	–	255[b]	107[c]	[illegible]
3	Dieldrin	1	240[d]	128	[illegible]
		5	137[d]	91	[illegible]
6	Dieldrin	1	200[d]	117	[illegible]
		5	91[d]	72	[illegible]
10	Dieldrin	1	107[d]	99	[illegible]
		5	50[d]	38	[illegible]
3	HCB	5	280[d]	148	[illegible]
		100	172[d]	72	[illegible]
6	HCB	5	248[d]	109	[illegible]
		100	103[d]	40	[illegible]
10	HCB	5	176[d]	77	[illegible]
		100	69[d]	19	[illegible]

[a] Splenic PFC/10^6 cells determined on day 4 following 10 infection of 0.1 ml of 10% SRBC. Malaria-infected mice (1 × 10[illegible] parasites, IP) were immunized on day 3 post infection, and Leishmania infected mice (1 × 10^6 promastigotes, ID) were immunized 2[illegible] days post infection.

[b] Control consisting of untreated but immunized mice.

[c] Control consisting of PFC responses in infected mice receiving no chemical treatment.

[d] Control consisting of chemically treated, on infected mice.

chemical treated, infected or chemical-treated plus infected mice as compared to control values (Table 4). The only statistically significant alteration occurred in the 5 ppm dieldrin-treated plus Leishmania-infected mice at the 10 week exposure period in which a significant decrease in macrophages, T-cells and B-cells was observed as compared to normal controls, infected animals, or 5 ppm dieldrin at the 10 week exposure period.

In contrast to the apparent absence of any consistent alteration in T-cell numbers in chemical-treated plus infected mice, a significant depression in HCB and dieldrin-treated plus Leishmania-infected mice to the T-cell mitogen, PHA, was demonstrated (Table 5). Isolated splenic T-cells from Leishmania-infected mice which had been administered dieldrin or HCB had a significantly depressed response to the polyclonal T-cell mitogen PHA. Neither dieldrin or HCB alone or malaria or Leishmania infection alone caused any change in the splenic T-cell enriched population response to PHA. Furthermore, malaria infection in dieldrin or HCB-treated mice

Table 3. Direct splenic plaque formation to PVP in mice infected with malaria or Leishmania with administered dieldrin or HCB.[a]

	Chemical treatment		Splenic PFC/10^6 cells		
Time, weeks	Agent	Concn, ppm	No infecting organism	Malaria	Leishmania
–	None	–	287[b]	180[c]	26[illegible]
3	Dieldrin	1	290	205	19[illegible]
		5	250	222	13[illegible]
6	Dieldrin	1	277	227	15[illegible]
		5	233	197	9[illegible]
10	Dieldrin	1	249	173	9[illegible]
		5	200	172	4[illegible]
3	HCB	5	269	293	17[illegible]
		100	241	214	14[illegible]
6	HCB	5	248	254	12[illegible]
		100	223	188	10[illegible]
10	HCB	5	257	231	8[illegible]
		100	189	153	7[illegible]

[a] PVP (Sigma) was administered IP at a dose of 2.5 μg/mouse to mice infected with *P. berghei* (1 × 10^5 parasites, IP[illegible] on day 4 post-immunization and to mice infected with *L. tropica* (1 × 10^6 promastigotes, ID) on day 20 post infection.

[b] Untreated, uninfected mice.

[c] Direct plaques were enumerated on day 4 post immunization, $n = 12$.

Table . Mononuclear (T, B, Mϕ) cells in mice infected with malaria or Leishmania and receiving dieldrin or HCB.[a]

	Chemical treatment			Mononuclear cells		
Time, weeks	Agent	Concn, ppm	Infecting organism	T, %	B, %	Mϕ, %
–	None	–	None	30	51	12
–	None	–	Malaria	34	60	15
–	None	–	Leishmania	31	58	13
3	Dieldrin	1	None	33	54	15
			Malaria	33	50	12
			Leishmania	30	50	15
3	Dieldrin	5	None	20	49	18
			Malaria	24	56	10
			Leishmania	32	47	10
6	Dieldrin	1	None	29	48	12
			Malaria	30	52	15
			Leishmania	24	41	12
6	Dieldrin	5	None	30	42	10
			Malaria	27	57	12
			Leishmania	24	40	8
10	Dieldrin	1	None	37	50	12
			Malaria	27	46	9
			Leishmania	23	37	10
10	Dieldrin	5	None	27	40	14
			Malaria	20	60	12
			Leishmania	19	41	5
3	HCB	5	None	30	49	12
			Malaria	32	54	12
			Leishmania	33	50	10
3	HCB	100	None	27	57	14
			Malaria	31	50	15
			Leishmania	30	46	9
6	HCB	5	None	28	51	15
			Malaria	32	51	14
			Leishmania	37	49	11
6	HCB	100	None	25	54	11
			Malaria	31	46	12
			Leishmania	29	40	11
10	HCB	5	None	34	47	10
			Malaria	30	47	16
			Leishmania	28	47	9
10	HCB	100	None	27	42	13
			Malaria	26	40	11
			Leishmania	27	48	9

[a] Mononuclear splenic cells (T-cells, B-cells, and macrophages) as mean percentages; $n = 12$. Cellular populations were enumerated on day 4 in malaria-infected mice (*P. berghei*, 1×10^5, IP) and day 20 in Leishmania-infected (1×10^6 promastigotes, ID) mice.

did not alter PHA responsiveness. The dieldrin + Leishmania-induced impairment in PHA activity was time- and dose-related, whereas the change seen in HCB + Leishmania-infected animals was only time-related, i.e., exacerbation with extended dietary administration of HCB.

To further evaluate the impaired PHA responsiveness in dieldrin-treated mice infected with Leishmania, the splenic T-cell enriched population was subjected to mitomycin C and anti-theta (anti-Thy-1) serum. Mitomycin C and anti-theta serum abolished the mitogenic response to PHA, as measured by ^{3}H-thymidine incorporation. When splenic T-cells taken from dieldrin + Leishmania infected mice were added at one-half the normal cell concentration (4×10^5) to normal (control) splenic T-cells a significant inhibition was demonstrated (Tables 6 and 7. This inhibition could be blocked by pretreating the dieldrin + Leishmania-infected mouse derived T-cells with mitomycin C which suggested an active cell-mediated suppressor. Similarly, when the splenic T-cells from the dieldrin + Leishmania-infected mice were pre-treated with anti-Thy-1 antiserum their inhibitory or suppressive effect was also abolished. These data suggested the presence of a Thy-1 positive suppressor cell in the spleen of the dieldrin-treated/Leishmania-infected mice.

Since the Leishmania promastigote has been described as a parasite infecting and proliferating in macrophages it was subsequently decided to de-

Table 5. PHA response of splenic T-cell nylon wool-enriched populations from mice infected with malaria or Leis nania and receiving dieldrin or HCB.[a]

	Chemical treatment		PHA response, cpm × 10^3		
Time, weeks	Agent	Concn, ppm	No infecting organism	Malaria	Leis ıania
–	–	–	39	41	[illegible]
3	Dieldrin	1	48	43	[illegible]
		5	53	39	[illegible]
6	Dieldrin	1	49	37	[illegible]
		5	45	41	[illegible]
10	Dieldrin	1	41	40	[illegible]
		5	44	36	[illegible]
3	HCB	5	54	36	[illegible]
		100	50	37	[illegible]
6	HCB	5	42	39	[illegible]
		100	41	41	[illegible]
10	HCB	5	37	35	[illegible]
		100	38	33	[illegible]

[a] Splenic cells were isolated from malaria- and Leishmania-infected mice on days 4 and 20, respectively, post infectio and eluted from a washed nylon wool (Fenwal) column (60 ml syringe barrel) with RPMI 1646 with 5% ICS at a rate of 1 ml/min. PHA Difco) was added at a concentration of 0.1 μg/well containing 200 μl of a 2 × 10^6 cell/ml suspension. All plates (Flow, Linbro mic test) were inoculated with PHA for 48 hr with 0.1 μCi of ^{3}H (5 Ci/mmole) added 16 hr before harvest.

Table 6. PHA response of mitomycin C-treated splenic T-cell enriched population from Leishmania-infected mice administered dieldrin.[a]

Cell origin	PHA response, cpm × 10^3
Normal	43
Normal + mitomycin C (20 μg/ml)	2
Leishmania (d. 20)	44
Leishmania + mitomycin C	6
Dieldrin (5 ppm, 10 wk)	40
Dieldrin + mitomycin C	3
Dieldrin/Leishmania	10
Dieldrin/Leishmania + Normal	15
Dieldrin/Leishmania (mitomycin C) + Normal	26
Half number of normal	24
Normal + normal mitomycin C-treated cells	22

[a] Cell preparations were used as previously described in Table 5. The enriched T-cell population at a concentration of 1 × 10^7 cells/ml was incubated with 20 μg/ml of mitomycin C (Sigma) for 25 min at 37°C. The cells were washed three times with fresh medium and numbers adjusted for subsequent use. Each study was conducted in triplicate.

Table 7. PHA response of anti-theta treated splenic T-cell enriched population from Leishmania-infected mice administered dieldrin.[a]

Cell origin	PHA response, cpm × 10^3
Normal	49
Normal + anti-theta	4
Leishmania (d. 20)	43
Leishmania + anti-theta	5
Dieldrin (5 ppm, 10 wk)	48
Dieldrin + anti-theta	2
Dieldrin/Leishmania	12
Dieldrin/Leishmania + Normal	14
Dieldrin/Leishmania (anti-theta) + Normal	30
Half number of normal	21
Normal + normal anti-theta treated cells	20

[a] All cells were prepared as described. Nylon-wo passaged spleen cells were incubated with a 1:80 dilution anti-thy-l antiserum (Cederlane Labs) and a 1:10 dilutio of rabbit complement.

termine if macrophage dysfunction contributed to the induction of the Thy-1 positive suppressor cells. Hepatic Kupffer cells, alveolar, splenic and peritoneal macrophages isolated from dieldrin treated (5 ppm, 10 wk) Leishmania-infected (day 20) mice were cultured for 24 hr, after which their supernatant, following centrifugation at 250*g* at 4°C for 15 min, was added to cell cultures simultaneously with PHA. Supernatant from hepatic Kupffer cells, and to a lesser extent from splenic macrophages, but not aveolar or peritoneal macrophages, when added to either control T-cells or T-cells from Le hmania-infected mice did not alter their response o PHA, however, when added to T-cells from di drin- or HCB-treated mice or from dieldrin/Le mania-infected mice, the supernatant suppressed e T-cell response to PHA (Table 8). Supernatant f m macrophage populations from dieldrin-treate mice or Leishmania-infected mice did not alter the esponse to PHA. The suppressive activity was emoved from the supernatant when dialyzed against phosphate-buffered saline overnight at 4° .

The production by hepatic Kupffer cells and to a lesser extent splenic macrophages, of a su pressive

Table 8. **M crophage induced alteration of PHA response of splenic T- ll enriched population from Leishmania-infected mice administered dieldrin.**[a]

Cell origi	PHA response, cpm × 10^3
Normal	48
Normal + ɸ	46
Leishmani d. 20)	43
Leishmani Mɸ	49
Dieldrin (pm, 10 wk)	43
Dieldrin + ɸ	18
Dieldrin/L hmania	11
Dieldrin/L hmania + Mɸ	6
HCB (100 m, 10 wk) + Mɸ	15
Half numb of normal	25

[a] Cells w prepared as described. Hepatic Kupffer, alveolar, splenic an eritoneal macrophage were isolated from dieldrin-treated Le hmania infected (d. 20) mice and cultured in RPMI 1640 + 1 FCS for 24 hr. Their supernatant was added to lymphocyt ultures simultaneously with the PHA; 50 μl of the supernata was contained in each well.

factor which impaired T-cell responses to PHA was correlated with the ability of hepatic macrophage from dieldrin/Leishmania treated mice to process an antigen and to transfer an adequate immunogen to a recipient control animal. Similar alterations were observed with splenic macrophages but to a lesser degree (Table 9). The impaired antigen processing capability was correlated with dietary exposure time.

Discussion

Extensive interest in immunotoxicology has been generated within the past 5-6 years by the demonstration that various environmental chemical contaminants may, in laboratory situations, be immunosuppressive. However, apart from contact dermatitis, clinical evidence indicating environmental chemical-induced immune alteration has not been available; although several examples of autoimmune diseases generated by chemical exposure have been reported (*12*, *13*).

Experimental immunotoxicologic data have primarily focused on evaluating the immunosuppressive effect of various environmental chemical

Table 9. **Macrophage presentation of antigen in mice infected with malaria and Leishmania and receiving dieldrin or HBC.**[a]

Time, weeks	Chemical	Macrophage donor type	PFC/10^6 spleen cells		
			No infecting organism	Malaria	Leishmania
--	—	Peritoneal	110	90	79
		Splenic	150	105	83
		Alveolar	91	79	64
		Hepatic	77	56	43
3	Dieldrin (5 ppm)	Peritoneal	91	77	69
		Splenic	109	120	71
		Alveolar	82	84	74
		Hepatic	86	61	30
	HCB (100 ppm)	Peritoneal	99	93	86
		Splenic	130	101	80
		Alveolar	115	89	69
		Hepatic	79	77	32
6	Dieldrin (5 ppm)	Peritoneal	76	61	43
		Splenic	85	103	32
		Alveolar	70	79	58
		Hepatic	71	44	77
	HCB (100 ppm)	Peritoneal	68	89	61
		Splenic	117	90	63
		Alveolar	103	76	53
		Hepatic	58	84	26
1	Dieldrin (5 ppm)	Peritoneal	54	48	30
		Splenic	71	74	20
		Alveolar	54	87	37
		Hepatic	68	37	16
	HCB (100 ppm)	Peritoneal	61	77	32
		Splenic	102	79	56
		Alveolar	89	83	49
		Hepatic	41	50	20

[a] Macro ges were isolated from dieldrin (5 ppm)- and HCB (100 ppm)-treated mice infected with malaria or *Leishmania* on days 4 and 20, r ectively. SRBC were opsonized in 50% FCS for 30 min and labeled with $Na^{51}CrO_4$. A standardized inoculum of ^{51}Cr-SRBC was injec d IV into control BALB/c mice and PFC/10^6 cells were determined on day 4; $n = 10$.

contaminants, food additives, drugs and physical agents while, in general, disregarding the possible immunostimulation that may occur. In this regard, time and dose may be the critical determinants deciding whether a chemical contaminant is considered to be an immunostimulant or immunosuppressant. Furthermore, the catabolic capability of the lymphoid-macrophage elements at both a basal level and at an immune and/or stimulated level have been neglected.

Since the immune response of an organism is a complex multicellular and humoral event an evaluation of a chemical as an immunosuppressant should initially encompass an immune parameter which is a maximal determination of the host immune capacity. Such a measurement is best reflected in the assessment of the host defense using a challenge with a pathogen. The pathogen used may be a bacterium, virus or complex parasite. Indeed, a systematic evaluation of cell-mediated immunity and humoral immunity may be conducted using a series of various pathogens.

In addition to microbial pathogens, suitable parasitic models are also available. Commonly used parasites for immunological studies are: *Schistosoma mansoni*, *Trichinella spiralis*, *Plasmodium berghei*, *Toxoplasma gondii*, *Trypanosoma cruzi*, and *Leishmania tropica*. The present study utilized two of these parasites, *Plasmodium berghei* (NYU-2) and *Leishmania tropica*, to evaluate the influence of two common environmental chemical contaminants dieldrin and hexachlorobenzene (HCB) on host defense capacity and immune competence. *Plasmodium berghei* (NYU-2) is a lethal murine malaria which evokes a T-cell, B-cell and macrophage response. Although it is lethal, survival time is a reflection of immune competence. The *L. tropica* promastigote is a macrophage parasite which may or may not survive and proliferate in tissue macrophages depending on their functional status (*14*).

In the present study host defense mechanisms were more significantly impaired in dieldrin-treated mice than in mice receiving HCB. Indeed, the Leishmania model did appear to be a more sensitive indicator of immunotoxicity since mice bearing this parasite had a marked lethality whether treated with dieldrin or HCB (Table 1). However, immunosuppressive synergism to a T-dependent antigen, SRBC, in the parasite and chemical-treated mice was more evident in mice exposed to HCB than to dieldrin (Table 2). It appeared that the chemical and not the parasite was the determining factor in the antibody response to SRBC. However, when a T-independent antigen was used, i.e., PVP, the only statistically significant immunosuppressive synergism occurred in dieldrin and HCB-treated mice bearing the Leishmania parasite. Since the antibody response to PVP has been reported to be macrophage-independent (*15*) it would suggest that B-cells/plasma cells are the target cell type involved. Lack of an altered response to T-independent antigens in malaria infected mice has been reported. Since a significant decrease in splenic T-cells, B-cells and macrophages was only seen in the dieldrin/Leishmania treated mice in the 5 ppm–10 week group, a change in gross cellularity would seem not to be a causal factor. However, a functional alteration can obviously not be excluded.

In this regard, the marked synergism between Leishmania in both dieldrin- and HCB-treated mice in regard to their response to PHA denotes a very selective cellular impairment in T-cell functionality. Since the 5 ppm–10 wk dieldrin/Leishmania-treated group demonstrated the most consistent alteration in immune parameters, i.e., depressed SRBC response, depressed PVP responses, and decreased T, B and Mϕ numbers and depressed PHA response, this group of animals was examined in greater detail for cellular hypofunctionality.

Treatment of splenic T-cell enriched populations from the dieldrin/Leishmania treated mice with mitomycin C to inhibit DNA synthesis or with antiserum to the Thy-1 antigen blocked their ability to inhibit the PHA response of normal T-cells. The inhibitory or suppressing activity suggested the presence of Thy-1 positive DNA synthesizing T-cells. Since induction of T-suppressor cells by macrophages has been demonstrated (*16*) the contribution of macrophages from dieldrin/Leishmania to the induction of T-suppressor cells (T_s) was investigated.

Isolated hepatic, and to a lesser extent splenic, macrophages produced a dialyzable factor that when added with PHA to splenic T-cell cultures, suppressed PHA stimulation of the T-cells. This macrophage T-cell suppressor factor did not influence normal control cells nor T-cells from Leishmania infected mice. However, when added to T-cells from dieldrin/Leishmania mice or dieldrin or HCB-treated mice it inhibited their proliferative response. The inhibition seen in HCB derived T-cells suggests that the factor may act on organohalide-primed cells. Although activated macrophages have been demonstrated to secrete inhibitory factors (*17*) previous studies have shown that macrophages from organohalide xenobiotics are not activated (*4*). Indeed, as observed in Table 9, the inability of the macrophages to present an adequate immunogen to naive hosts indicates a suppressed state.

The demonstration that maximal suppressor factor was found associated with hepatic Kupffer cells may be, in part, related to hepatotoxicity of the two xenobiotics. If this is the case, it may provide a valuable tool in the study of the relationship between hepatotoxicity and immunosuppression.

Further studies to elucidate the factor produced by the suppressor macrophages will evaluate interferon production as well as prostaglandins which have immunomodulatory roles (*18*). In addition, macrophage secreted factors may also compete with thymidine for its uptake into DNA by T-cells.

The incorporation of infectious disease models into immunotoxicologic studies may be a more relevant as well as more sensitive indicator of immunotoxicity. Although most studies have utilized mature animals, the use of neonates and aged animals in conjunction with stress may be a more accurate measurement of immune potential.

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Environmental Health Perspectives
Vol. 43, pp. 99-107, 1982

Use of Experimental Airborne Infections for Monitoring Altered Host Defenses

by Donald E. Gardner*

The success or failure of the respiratory system to defend itself against airborne infectious agents largely depends upon the efficiency of the pulmonary defenses to maintain sterility and to dispose of unwanted substances. Both specific and nonspecific host defenses cooperate in the removal and inactivation of such agents. Several studies have shown that these defenses are vulnerable to a wide range of environmental agents and that there is a good relationship between exposure to pollutant and the impaired resistance to pulmonary disease. There are numerous immunological, biochemical and physiological techniques that are routinely used to identify and to characterize individual impairments of these defenses. Based on these effects, various hypotheses are proposed as to what health consequences could be expected from these effects. The ultimate test is whether the host, with its compromised defense mechanisms, is still capable of sustaining the total injury and continuing to defend itself against opportunistic pathogens. This paper describes the use of an experimental airborne infectious disease model capable of predicting subtle changes in host defenses at concentrations below which there are any other overt toxicological effects. Such sensitivity is possible because the model measures not just a single "health" parameter, but instead is capable of reflecting the total responses caused by the test chemical.

Introduction

The lung, being a prime target organ to environmental insults, has been shown to be vulnerable to a wide range of noxious substances, some of which can cause serious dysfunction of normal respiratory (gaseous exchange) and/or nonrespiratory (host defenses, metabolism) functions of the lung. Such adverse effects would be expected to have numerous ramifications on the host and increase the unwanted risk of disease.

Under normal conditions the lung has the capability to maintain pulmonary sterility and disposal of unwanted substances by constantly removing or inactivating any such agents that are deposited in the respiratory tract. This is usually successfully accomplished through the cooperative efforts of both specific and nonspecific host defenses. A major challenge for the pulmonary toxicologist is to identify and to describe individually each and every subtle impairment of these pulmonary defenses caused by the inhaled chemicals. Such a comprehensive assessment, although relevant, would be costly, time consuming, difficult for routine toxicological testing and may not individually be sensitive enough to provide the necessary data base for establishing safety of the test compound.

The purpose of this discussion is to describe a much simpler approach for monitoring such subtle changes using a whole animal model and to illustrate the validity and the use of such a biological screening system for standard toxicological testing. The success of this test system depends upon detecting the ability of the host to cope with the total injury induced by the inhaled substance. With this testing procedure, animals with normal pulmonary defenses are quite capable of defending the lung against such assaults, but significant alterations in any of the several pulmonary defenses would be

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expected to prolong microbial viability and to enhance the establishment of the potentially hazardous microorganisms, thereby increasing the risk of pulmonary disease.

The Microorganisms

Although a wide range of infectious agents, both bacterial and viral, has been successfully employed by various investigators, we primarily use a nonhuman pathogen (*Streptococcus pyogenes*, group C) that was originally isolated from a spontaneously infected pharynx of a guinea pig in our laboratory. These bacteria are maintained either lyophilized or on brain-heart infusion agar and are periodically passed in mice to maintain their virulence. Prior to the day of the test, the organism is grown for 24 hr in Todd-Hewitt broth, and the concentration of organisms adjusted spectrophotometrically so that approximately 1 × 10^9 organisms per exposure are aerosolized. Quantitative studies of viable agents have illustrated that such organisms may undergo considerable destruction during the aerosolization processes (*1*). As would be expected, the degree of lability varies with the microorganism. Factors responsible for this destruction may include shearing force, impaction, agitation, dehydration, and possibly some oxidative effects occurring during aerosolization.

If the infectious model system is to be an effective screen for determining the adequacy of the host's pulmonary defenses, the infectious agent must fulfill certain criteria. It must remain alive and infectious in aerosol; be deposited and capable of multiplying in susceptible tissue; have minimal influence on the mortality rate when small variations in dose or virulence occur; have low natural mortality in the normal host; have a method available for quantifying the bacteria dose; elicit reproducible quantitative data; and be nonpathogenic for humans for safety reasons.

Experimental Airborne Infection

The exposure methodology for this test system is shown schematically in Figure 1. Test animals are

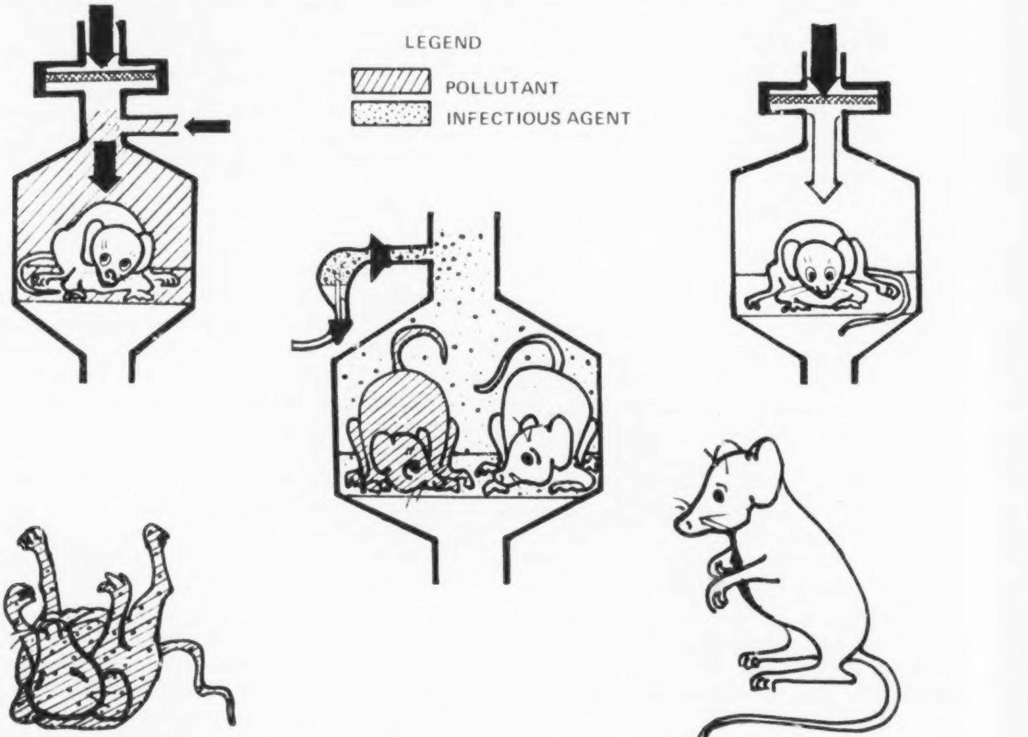

FIGURE 1. Schematic drawing of infectivity model system. Animals are exposed to either clean air or test chemical. After this initial exposure the two groups are combined and exposed to an infectious microorganism. The data is expressed as percent mortality between the test groups.

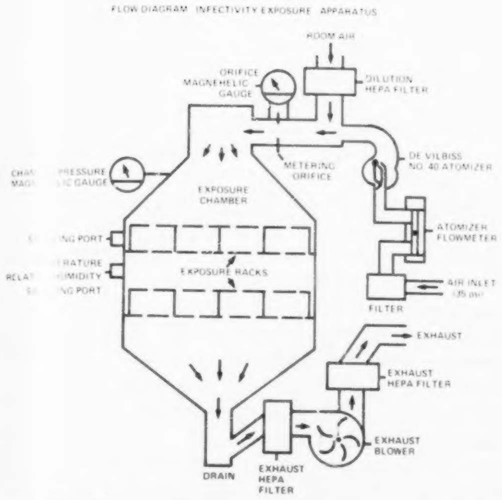
FLOW DIAGRAM INFECTIVITY EXPOSURE APPARATUS
ROOM AIR
ORIFICE MAGNEHELIC GAUGE
DILUTION HEPA FILTER
DE VILBISS NO. 40 ATOMIZER
CHA PRESSURE
MAG IC GAUGE
METERING ORIFICE
EXPOSURE CHAMBER
ATOMIZER FLOWMETER
ING PORT
RATURE
RELA UMIDITY
NG PORT
EXPOSURE RACKS
AIR INLET (35 psi)
FILTER
EXHAUST
EXHAUST HEPA FILTER
EXHAUST BLOWER
DRAIN
EXHAUST HEPA FILTER

FIGURE 2. Diagram of the chamber exposure system for inhalation exposure to an infectious microorganism (Rochester type).

randomly selected to be exposed to either clean air or to the test substance. The operational procedures for this exposure, including the generation of exposure atmospheres, sampling and characterization methodology, and experimental design for delivering the airborne test materials, will be dictated by the objectives of the particular toxicologic investigation. The duration of the exposure and the mode of exposure, i.e., whole body, head only, nose or mouth only or lung only, can all be employed in this model system.

The methods developed for operation of the infectious exposure chamber and the exposure of animals should be designed to insure the safety of the scientific personnel. It should be remembered that many microorganisms are potentially pathogenic and that standard microbiological safety techniques must be employed in these operating procedures.

At various times after the exposure to the test chemicals, both the pollutant exposed and control animals are combined and placed in another chamber for a 15 min exposure to an aerosol of infectious organisms. A flow diagram of the infectivity chamber is given in Figure 2.

Atomization of Infectious Organisms

The production of an evenly dispersed cloud of infectious agents under controllable conditions is fundamental for experimental studies of airborne infection. Considerable research has been conducted concerning the methodology for atomization of the infectious organisms (*2-4*). Atomizers are devices used to produce a continuous finely divided particle or droplet from a liquid suspension or solution. Although a number of different types of generators are available to disperse liquids to form aerosols (spinning disc atomizers, ultrasonic nebulizer, and vibrating orifice generators), we have found the DeVilbiss No. 40 compressed air nebulizer (*5*) to be highly efficient (Fig. 3).

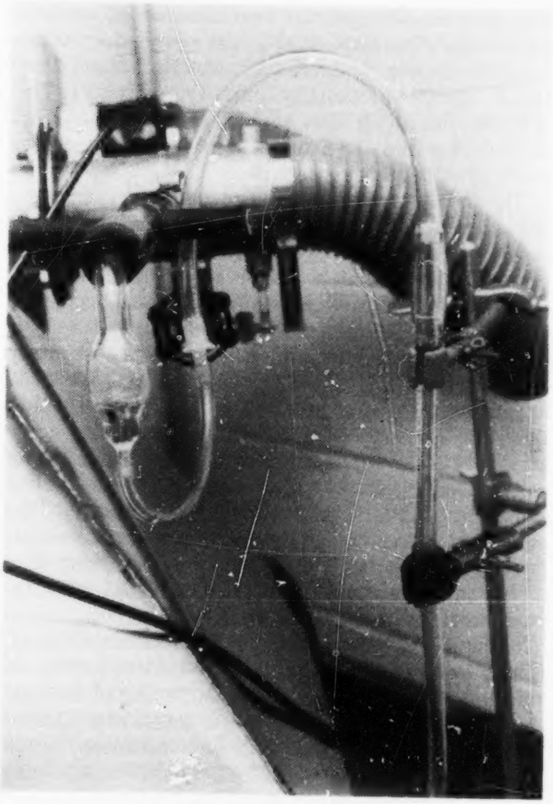

FIGURE 3. DeVilbiss No. 40 compressed air nebulizer for generation of microbial aerosol.

These generators atomize a liquid by moving a blast of air across a narrow nozzle into which liquid has been drawn by the Bernoulli effect. Large droplets impinge on the upper wall of the exit duct while the smaller droplets remain aerosolized. The size range of the primary droplets is from 1 to 7 μm with a MMD of 1.3. The outputs are in the order of 1–30 μl of fluid/liter of air. A 5-ml portion of the fluid containing the infectious organisms is atomized over a 15-min period. Air continues to pass through the "dried" nebulizer for an additional 5 min (air wash) before the test animals are removed from the chamber. In order to maximize the reduction in variability in total dose deposited in the respiratory tract, the animals are randomized in stainless steel mesh racks which individually partition animals. This prevents huddling and aids in insuring that each animal receives as nearly as possible, the same number of viable organisms.

The measurement of the concentration of microorganisms within the chamber fails to adequately define the actual viable dose delivered to the target organ. Therefore it is important to measure pulmonary deposition of the viable agents. This can be accomplished if, immediately following this aerosol exposure, two to four animals from each test group are sacrificed and the lung and trachea removed and completeiy homogenized in sterile broth. Aliquots of the homogenized sample are cultured in triplicate on blood agar plates and total number of colony-forming units (cfu) of viable organisms determined by standard microbiological techniques. The same techniques can be used at other time intervals during the experiment to provide comparison data on *in vivo* clearance (i.e., bacteriocidal activity) mechanism of the various treatment group.

For maximum sensitivity, it is important to maintain a low mortality rate in the control group. In this way, a slight shift in the functioning of the host defenses can be measured in the terms of enhanced mortality. However, day to day variation in the number of organisms initially deposited in the lung can be routinely expected. For this reason it is necessary to know the relationship between number of viable organisms deposited and the resulting mortality. This can be accomplished by examining the cumulative data from all of the contro mice. Figure 4 illustrates, that with our organis . one can expect that, within the range of 200-4000 cf lung, there is no apparent correlation between nur er of bacteria deposited in the lung and resulting ii rease in percent mortality. However, at higher le ls of bacterial deposition one can expect that the ol rved mortality will increase further (*6,7*).

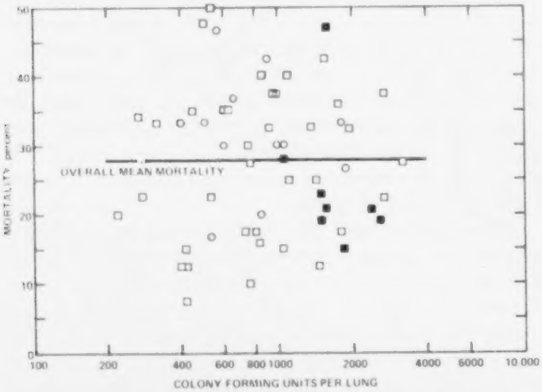

FIGURE 4. Correlation between the number of viable organisms deposited in the lungs of control mice and the resulting percent mortality. (■), (□), and (○) represent the data from experiments which used 100, 40 and 30 control mice, respectively (*7*). The correlation coefficient (0.002) shows no relationship between these variables.

After receiving the bacterial challenge, t test animals are separated into their appropriat reatment groups and maintained in clean air r 15 days, during which time the mortality ra are determined. With streptococci, the typical ex cted mortality rate in the control group range from 10-30%. This mortality reflects the natural sceptibility of the host and the virulence of ti test microorganisms.

Selection of Test Animals

To maintain the health of the individual test animal, many of the host's available defenses must be able to act rapidly and efficiently if they are to limit the extent of the induced laboratory infection. In the resistant animal, the host defenses are functionally capable of quickly reducing the activities of the organism in the tissue. The unhampered lung, with all of its bactericidal capability can, within hours, restore the sterility of its environment (*8-10*).

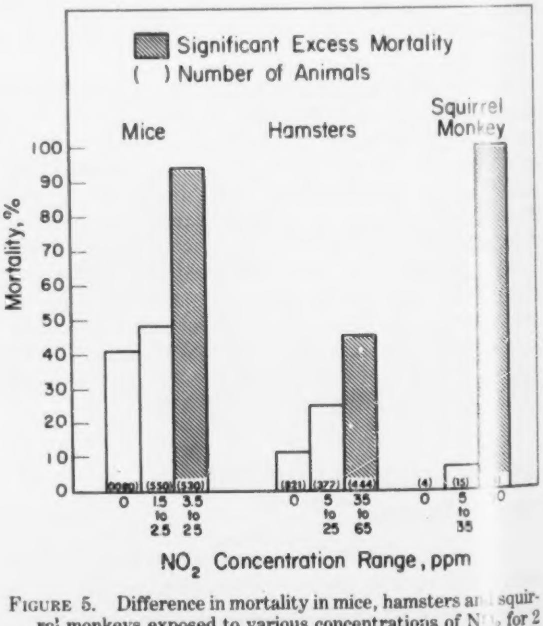

FIGURE 5. Difference in mortality in mice, hamsters a squirrel monkeys exposed to various concentrations of N $_2$ for 2 hr and subsequently challenged with an infectious ag t (*15*).

Table 1. Mortality in different mouse strains after exposure to 5 ppm NO_2 and challenge with Klebsiella aerosol.[a]

Strain	Air D/T	Air %	NO_2 D/T	NO_2 %	Change, %
BDF ack)	31/120	25.8^b	40/120	33.3	7.5^b
C57B (black)	24/70	34.3^c	36/70	51.4	17.1^c
Swiss lbino)	164/390	42.1^c	262/390	67.2	25.1^d
Balb bino)	48/100	48.0^c	72/100	72.0	24.0^d

[a] Means in a column with the same superscript are not significantly different ($p \leq 0.05$).

[b] Significantly different from controls ($p \leq 0.05$).

Table 2. Number of replications needed to detect various mortality increases as a function of the number of mice per group.[a]

		Mortality difference, (treated-control), %		
Mice/group	SD	7.5	1.5	30
20	0.1034	31	9	3
30	0.09	19	6	2
40	0.071	15	5	2

[a] Type 1 error = 0.05, type II error = 0.2.

The ability to resist infection with any given organism is variable not only between species but also within species. Although we use primarily 4-6-week-old female CD-1, COBS mice, a variety of other animal species have been employed with the infectivity model system including the squirrel monkey, rat, hamster, guinea pig, and mouse (*11-14*). The choice of the species may vary with the nature of the information sought. A degree of caution is warranted when one tries to use this model system for comparing the effects of a given chemical on different species of animals. Figure 5 shows the response of mice, hamsters and squirrel monkeys to exposure to NO_2 and subsequently challenged with *Klebsiella pneumoniae* aerosol (*15*). These data would suggest that either the squirrel monkeys are more resistant to NO_2 than mice, since it took significantly more NO_2 to produce the observed effect, or they are less susceptible to that particular infectious agent. Such information is essential in regulatory settings. However, when one compares the mortality rate within the control animals, it indicates that the animal's natural resistance to the bacterial pneumoniae was quite different for the different species. The respective mortality rates in the control mice, hamsters and monkeys were 41%, 11% and 0%. This illustrates the importance of the selection of the proper organism for testing as well as indicating that one needs to be careful in interpreting the data.

Difference in natural resistance to the airborne infection has also been seen within different strains of mice (*15*). Table 1 shows the mortality in four different strains of mice after exposure to NO_2 and challenged with *Klebsiella pneumoniae* aerosol.

Statistical Considerations

Since there is variability between replications of the individual experiments due to both number and the virulence of the viable microorganisms aerosolized and subsequently deposited within the lung, it is important that the data be examined using sound statistical analysis for reducing the experimental error. This can be achieved by expressing the data for each replication as the difference in mortality between the chemically exposed group and the air controls or by probit analysis using a correction for natural mortality. Table 2 provides an example, using our system, of the recommended number of replications necessary to detect percent mortality differences based on the number of mice per group. The clearance of the microorganisms from the lung can be analyzed by an analysis of variance which accounts for the assay error of the microbiological technique.

Effects of Airborne Metals and Particulates

Evidence exists which indicates that *in vivo* and *in vitro* exposure to a number of trace metals can significantly alter the basic functioning of several host defense systems (*16-20*). In many cases, these insults can be correlated to significant increases in susceptibility to infection. Inhalation of metals such as Ni and Cd affects not only the phagocytic and enzymatic activity of the alveolar macrophages, but these metals also affect the primary humoral immune system (*21-24*).

Figure 6 shows the dose-response relationship of various trace metals when tested in the infectivity system. It is important to notice that neither Fe_2O_3 nor carbon dust produced any significant effects. This indicates that the model system can distinguish between particulates which are inert and

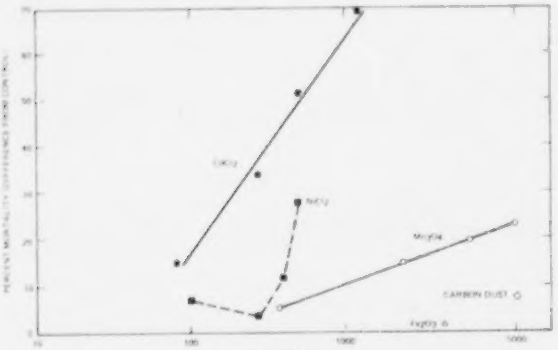

FIGURE 6. Increase in susceptibility to pulmonary infections following a 2 hr exposure to various airborne particulates.

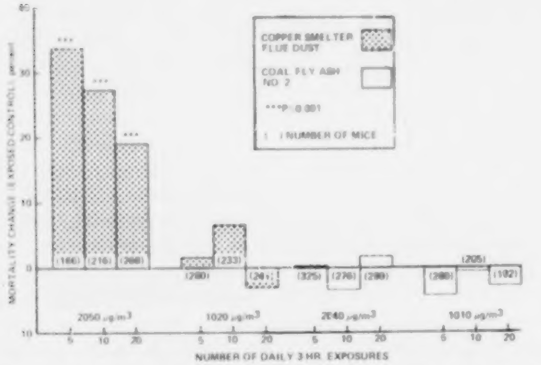

FIGURE 7. Effects of multiple daily 3 hr exposure to aerosols of copper smelter dust or coal fly ash No. 2 on susceptibility to respiratory streptococcus infection (*25*).

those that are potentially toxic. In this study, the toxicity ranking for these metals was Cd > Ni > Mg. Ehrlich (*15*) also used this model system for comparing the toxicological effects of a number of sulfate compounds. From his studies, he estimated that concentrations of the compound (3 hr exposure) which produced a 20% excess in mortality over controls were: 0.4 mg/m³ $CdSO_4$, 1.0 mg/m³ $CuSO_4$, 2.5 mg/m³ $ZnSO_4$, 2.6 mg/m³ $Al_2(SO_4)_3$ and 3.8 mg/m³ $Zn(NH_4)_2(SO_4)_2$. Concentrations as high as 8 mg/m³ of NH_4NO_3, KNO_3, NH_4HSO_4, $(NH_4)SO_4$, and Na_2SO_4 did not result in any significant changes in mortality.

In addition to testing of pure compounds, this system has also been used to evaluate "real world" industrial particulates collected from copper smelter and from a fluidized-bed coal fired power plant (*25*). Mice were exposed for 5, 10, or 20 daily 3-hr (5 days per week) exposures prior to receiving the streptococcal infection. Figure 7 illustrates the excess mortality observed in the copper sample as compared with the coal fly ash. In these studies, Aran et al. (*25*) were successful in correlating the m tality effects with some adverse effects on alveolar acrophages that could account for this incre e in susceptibility.

Effects of Gaseous Pollutant

In the past, a number of investigators had r orted that during chronic inhalation studies there vas a significant increase in spontaneous pneum ia in the animals being exposed to gaseous te sub-stances (*26,27*). These responses were su antiated under controlled laboratory experime ation where individual gases such as O_3 and NO_2 well as complex mixtures such as irradiated auto e aust, H_2SO_4 and O_3; O_3 and NO_2; O_3, SO_2 and hy rocarbons; were tested using the infectivity s tems (*28-32*).

The effects of various exposure regimen have also been investigated. For example, usi g the

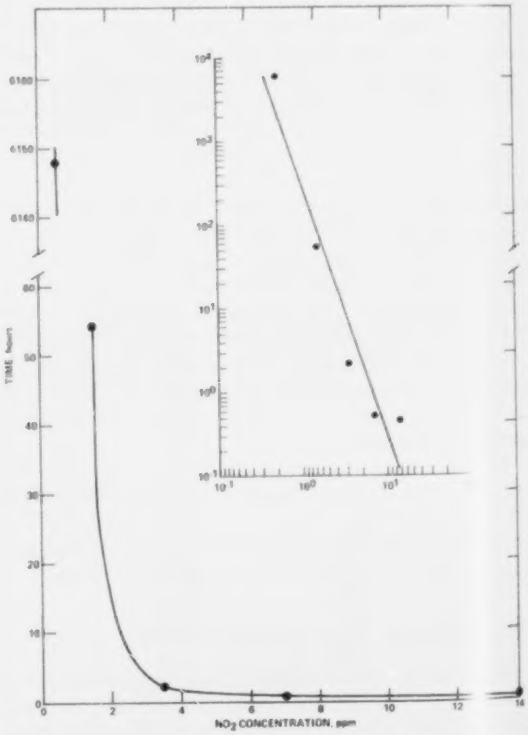

FIGURE 8. Time necessary to elicit a 20% mortalit n mice versus concentration of NO_2. The regression sh s that concentration-time relationship for mortality can b repre-sented by a straight line on a log-log scale.

Table 3. Percent excess mortalities in mice exposed for 3 hr to pollutants and challenged with streptococcus over corresponding infected controls.

NO_2 concentration, mg/m^3 (ppm)	O_3 concentration, mg/m^3 (ppm)			
	0	0.1 (0.05)	0.2 (0.1)	0.98 (0.5)
0	0	5.4	7.2	28.6[a]
2.82 (1.5)	−1.7	4.6	4.2	23.9[a]
3.76 (1.5)	14.3[a]	22.0[a]	--	56.2[a]
6.58 (1.5)	28.2[a]	--	38.5[a]	68.7[a]
9.40 (1.5)	35.7[a]	--	--	65.3[a]

[a] Significant change in mortality over controls ($p < 0.05$).

enhancement of mortality as the biological endpoint for measuring the toxic effects of NO_2, studies have been conducted which varied the exposure time from a few minutes to several months (*33*). Different concentrations ranging from 0.94 mg/m^3 to 526 mg/m^3 (0.5–28 ppm), different exposure modes (i.e., continuous vs. intermittent) and various concentration × time effects were evaluated. Figure 8 is a concentration-time ($C \times T$) curve using the endpoint of a 20% mortality enhancement with this model system. The regression obtained shows that the $C \times T$ relationship for mortality can also be represented by a straight line on a log-log scale when the exposures were continuous.

The infectivity model system has also been employed to evaluate the effects on the dose-response relationship when two gases were combined (*30*). In this study the animals were exposed for 3 hr to NO_2 alone, O_3 alone, NO_2 and O_3 or clean air. The data in Table 3 clearly indicate that these two gases produce an additive effect since the differences in mortality rates were, in most cases, equivalent to the sum of those resulting from each of the individual pollutants.

Simplifying the Model System

In order to be able to increase the utilization of this model system and to make it more applicable for general screening of a large volume of chemicals, it would be advantageous to simplify the exposure system. Since it has been generally recommended that the test agent be administered by the route that most closely simulates the major route by which human exposure occurs, it is important for toxicological studies of airborne chemicals that the primary target organ continues to be the lung. However, since the use of sophisticated aerosol exposure techniques are extremely costly and are not readily available to most laboratories, it would be useful if an alternative method of exposure were available. The intratracheal injection (IT) method is a convenient, acceptable and easy-to-use approach for testing hazardous substances. This approach has several advantages over inhalation in that relatively small amounts of the test substances are required; delivered dose can be accurately measured; large size particles can be tested; skin contamination is avoided; fewer safety and engineering problems are encountered; large quantities can be introduced into the respiratory system provided the substances are not too toxic; and much less expense is involved than required to establish an inhalation facility for generating and monitoring the test aerosol.

The possibility of combining IT instillation, as the route of exposure of the test substance, prior to the infectious challenge has been tested in our laboratory by Hatch and co-workers (*34*). This modification would increase the possible utilization of this infectious model system providing there was good correlation between the effects seen following the IT injection and those observed in inhalation studies of the same substances.

In these investigations, 23 compounds were initially tested. Each substance was injected IT in concentrations which were calculated to approximate the dose of the chemical which would be deposited in the lung as reported for inhalation studies. Comparison of the dose-effect curves of these two routes of administration indicates there was a general agreement between results of inhalation and IT injection studies.

Four of the chemicals tested that showed significant increased mortality (> 40%) by inhalation also showed similar mortality increases when the test substance was administered by IT instillation at levels calculated to be equivalent to those achieved during inhalation. Five of the chemicals caused small 20-30% enhancement by intratracheal, but not all produced an effect by inhalation. The remaining 14 compounds which showed no significant effect by inhalation also produced no effect when given by IT. Table 4 gives a representative sample of the data from this study illustrating the correlation between these two methods of administration (*34*). Although additional testing is required, the present data for

Table 4. Comparison of inhalation and intratracheal routes of exposure on enhancement of bacterial infection.[a]

Excess mortality ($p \leq 0.05$), %	Chemicals	Lung dosage, µg/mouse lung	
		Inhalation[b]	Intratracheal
> 40	$CdCl_2$	0.44	0.46
	$CdSO_4$	1.12	1.30
	$CuSO_4$	0.81	0.70
20–30	$AlNH_4(SO_4)_2$	1.82[c]	2.10
	$ZnSO_4$	1.40	0.7
No effect	$Ca(NO_3)_2$	2.29	2.10
	Carbon black	2.29	3.50
	Fe_2O_3	1.03	3.50
	$Pb(NO_3)_2$	3.30	5.61
	NH_4NO_3	3.60	2.10

[a] From Hatch et al. (*34*).

[b] Estimation of quantities of chemical per lung following inhalation.

[c] No significant effect with inhalation.

metals indicate that similar effects on susceptibility to lung bacterial infection can be determined whether the chemicals are deposited in the lung by inhalation or by intratracheal injection. Thus, IT injection appears to be a reliable method for establishing the likelihood of pulmonary toxicity of inhaled chemicals.

Summary

A reliable toxicological test should have the sensitivity to demonstrate a failure in some system to perform a normal function. In the infectivity models, the inability of the animals to defend themselves against opportunistic pathogens is the ultimate test of the hosts' defenses. Although the alveolar macrophages appear to be the major initial line of defense against these viable agents, there is evidence that local, specific immune responses also occur in the lung. Unfortunately, the actual mechanisms describing the interaction of these defense cells and the local humoral and cell-mediated immunity in protecting the host against such infectious insults is not yet clearly defined. In evaluating the usefulness and reliability of this whole animal model for predicting altered host defenses, several things can be considered. First, there is a possibility that a similar effect may occur in man, providing the microorganism present is capable of exploiting any alterations in host defenses and that the concentration of the inhaled pollutant reaching these defenses is sufficient to cause an adverse effect. Secondly, the model can also be used to provide information on the pathogenesis of infectious disease, and aid in understanding how the lung protects itself against various environmental challenges. Finally, because of the unique sensitivity of this infectious model, it is capable of identifying subtle changes at exposure concentrations that do not produce any other overt toxicological effects such as morphological changes, edema or gross physiological alterations. This last item is an important consideration when one is developing and evaluating various tests for possible use in screening applicability. The ideal test should be one that produces few false positives or false negatives, is predictive of human effects and be capable of measuring not just a single host response, but instead capable of reflecting the total responses caused by the test chemical resulting in subtle alterations to many functional systems present in the whole organism.

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Environmental Health Perspectives
Vol. 43, pp. 109-113, 1982

New Approaches to Immunotoxicity Testing

by Douglas L. Archer*

New approaches to immunotoxicity testing are reviewed and discussed. A method of activating T-cells *in vivo* is presented which circumvents artifacts due to viability effects encountered with *in vitro* mitogen assays. The use of adoptive transfer approaches to combine the advantages of *in vitro* manipulation with *in vivo* function assays is discussed relative to natural killer cells. The need for an *in vitro* metabolic activation step coupled to other *in vitro* immunologic assays is discussed.

Introduction

Previous papers have demonstrated the importance of the immune system with regard to the well-being of all animals. It has also been clearly demonstrated that chemicals can modify the immune response, some for the benefit of the animal, e.g., immunopharmaceuticals, and some in potentially harmful ways, e.g., chemical wastes and environmental pollutants. Adequate coverage of the new approaches to immunotoxicity testing would require a separate symposium. The volume of new methods being applied to all sorts of immunologic investigations is enormous, and the list of literature reports increases monthly. Thousands of compounds and their effects on the immune system require examination and rapid, economical screening procedures would certainly benefit industry as well as health and regulatory agencies.

General Approaches to Testing for Immunotoxicity

Table 1 shows the basic ways in which immune response tests may be carried out. In the first category of tests the cells involved in a particular immune response are exposed directly to the chemical *in vitro* and the test for immune function is also carried out *in vitro*. Although this approach is the most economical in terms of time and animals, it has some problems, most notably: metabolic alteration of the compound may not be accounted for; distribution of the chemical into the immune compartment is assumed; and cells are more susceptible to damage in the relatively dilute *in vitro* microenvironment. A compromise approach in terms of realism and economy calls for *in vivo* exposure to the chemical followed by *in vitro* testing of immune function of various lymphoid tissue. This is clearly the most common approach currently used in immunotoxicity studies. The major advantages of this approach are that distribution of the chemical to the immune compartment is realistic and metabolism of the compound may occur naturally. This approach will be discussed further. Lastly, animals may be exposed to the chemical *in vivo* and immune functions tested *in vivo*. Although this is the most

Table 1. General immune response test scheme.

Toxicant exposure	Test environment
In vitro	*In vitro*
In vivo	*In vitro*
In vivo	*In vivo*

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realistic approach, it too has definite drawbacks: large numbers of animals are usually required, owing to the variability of immune responses even in syngeneic populations of mice; and at present many of the *in vitro* manipulations and tests cannot be duplicated *in vivo*.

Returning to the *in vivo* exposure/*in vitro* analysis scheme, some disadvantages with this approach have recently been noted.

Lymphoproliferative Assays

Figure 1*a* depicts the standard lymphoproliferative assay (or mitogen assay) as currently performed. Although chemical contact occurs *in vivo*, it was thought that preparation of cells for *in vitro* culture might remove the chemical; thus chemical and activator (or mitogen) might not be present simultaneously. Conversely, cells might be metabolically or physically damaged by the chemical, making them appear nonfunctional in the more hostile *in vitro* situation, but compensatory mechanisms *in vivo* might permit normal functions to occur. To test these hypotheses, we used staphylococcal enterotoxin A (SEA), a T-cell specific mitogen (*1*) with low toxicity to the mouse and with the added advantage of a broad plateau of activation rather than the sharp dose-related peaks commonly seen with the lectin mitogens concanavalin A or phytohaemmagglutinin (*2*). SEA was shown to be an *in vivo* T-cell activator (*3*). The only *in vitro* culture required was a 4-hr period for ^{3}H-thymidine uptake (Fig. 1*b*). Thus, chemical and activator could be present simultaneously *in vivo*. During these studies, we noticed that certain agents administered *in vivo*, such as antithymocyte serum (ATS), which disrupts T-cell membrane integrity, showed a greater suppressive effect on *in vitro* mitogen responsiveness at far lower doses than were required to suppress *in vivo* activation of T cells by SEA.

Table 2 shows the effects of ATS on *in vitro* survival of cells, as determined by vital dye staining. Spleen cells from ATS-treated animals were 97-98% viable at the time of harvest. Four hours of culture had only a slight negative effect on *in vitro* survival of ATS-treated cells. The rate of cell death of 48-hr cultured cells (the routinely used LP assay time) was greatly accelerated by ATS pretreatment compared with the *in vitro* death rate of controls. Magnification of the apparent damage in the *in vitro* culture is advantageous because greater sensitivity is attained; however, further work has shown that realism may be compromised. Experiments have indicated that the shorter the *in vitro*

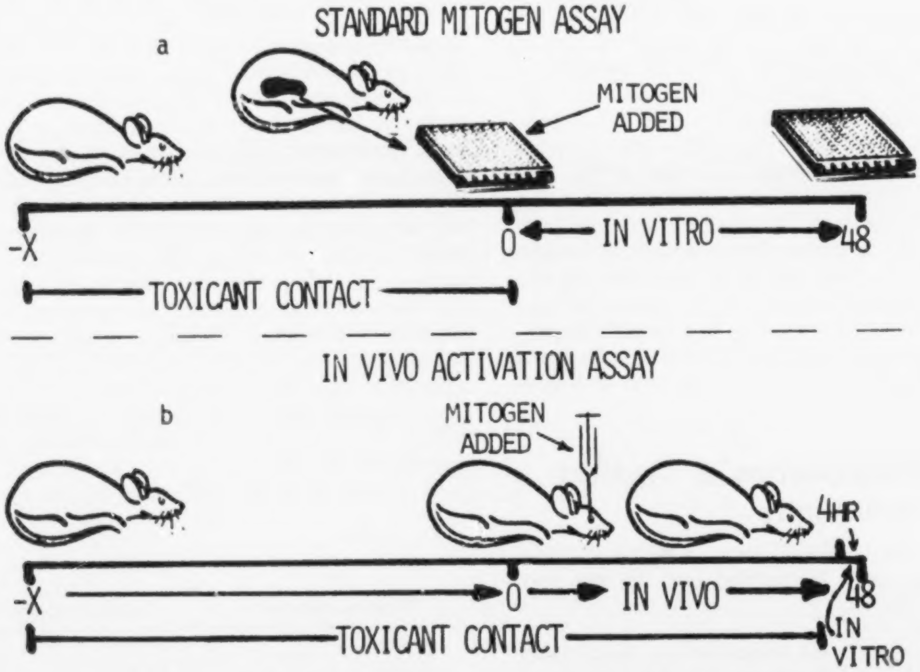

FIGURE 1. (*a*) Standard lymphoproliferative assay. (*b*) *In vivo* activation assay (*3*).

Table 2. Viability of ATS-treated and untreated mouse spleen cells after 4 and 48 hr *in vitro*.

Incubation time *in vitro*, hr	Viability by trypan blue dye exclusion, % (mean ± SD)[a]	
	Control for ATS	ATS-treated[b]
4	97 ± 1.1	95.2 ± 1.5[c]
8	84 ± 5.1	50.7 ± 7.9[c]

[a] $n = 12$ for each point

[b] Treatments carried out as described in text.

[c] $p < 0.01$ by Student's *t*-test performed on arc-sines of proportion of viability.

incubation time, the less the chance of amplified effects on viability.

In Vitro Manipulation-Adoptive Transfer

In a recent report concerning natural killer cells, Kasai et al. (*4*) demonstrate how the concept of short *in vitro* culture time might be applied. Natural killer (NK) cells have become the focus of many recent studies on antitumor activities of the immune system because they appear to be one of the first-line defenses against virus- or chemical-transformed cells and have a functional counterpart in humans. Kasai et al. attempted to demonstrate that cell subpopulations which expressed NK activity *in vitro* were actually responsible for NK activity *in vivo*. The antitumor role of NK cells *in vivo* had not yet been conclusively shown. The experimental approach taken by these investigators demonstrates how several new technologies could be applied to immunotoxicologic investigations. Figure 2 depicts the separation scheme used by Kasai et al. Their procedure, which could be applied to chemical-exposed animals as well, was as follows.

Spleen cells were removed from mice. Some cells were placed in plastic dishes coated with antimouse Fab antibody; in this way, immunoglobulin (Ig)-bearing cells (B-cells) were enriched and non-Ig-bearing cells were removed. These populations were retained for testing. Other spleen cells were passed through a nylon wool column known to retain Ig-bearing B-cells. Thus, T-cells bearing Thy 1 antigen were enriched as were other non-Ig-bearing cells. Some of these cells were treated with anti-Thy 1.2 antibody + C′, to specifically lyse the mature, Thy

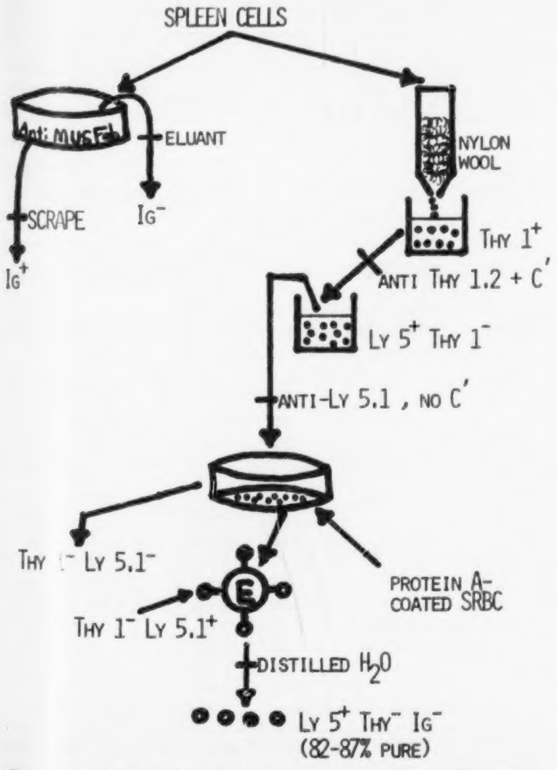

FIGURE 2. Separation of spleen cell populations used by Kasai et al. (*4*).

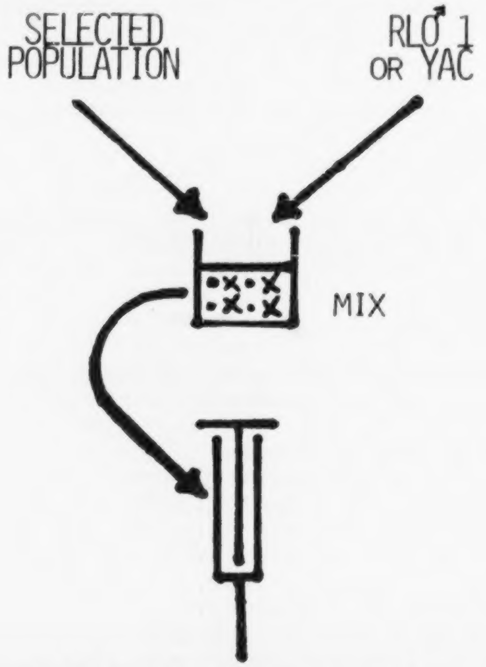

FIGURE 3. Adoptive transfer of tumor cells and separated spleen cell subpopulations used by Kasai et al. (*4*).

Table 3. Ability of different lymphocyte sets to prevent lymphoma growth *in vivo*.

Spleen cell population	Cell number (× 10^6)	Tumor incidence[a]	
		Host = BALB/c, tumor = RLol	Host = A, tumor = YAC
A None	--	22/22	16/16
B Unselected	10	8/8	8/8
C Nylon-passed cells	5	14/15	15/15
D Thyl$^+$ cells	5	8/8	8/8
E Ig$^+$ cells	5	16/16	16/16
F Thyl$^-$ cells	5	19/22	22/22
G Thyl$^-$Ig$^-$Ly5$^+$ cells	5	1/12	0/12
H Group G treated with anti-Ly5.1 + C	5	7/8	7/8

[a] Results of three individual experiments (*4*).

1-bearing T cells. Monoclonal antibody technology currently assures the purity of such antibody preparations. The Thy 1^- cells were found to have Ly 5 surface antigen by fluorescent antibody methods. The Thy 1^- Ly 5^+ cells were coated with anti-Ly 5 antibody, but without complement because no lysis was desired. The coated, viable cells were placed in dishes with protein A-coated sheep erythrocytes. Protein A specifically binds to the Fc portion of antibody molecules; the Ly 5^+ cells were bound to erythrocytes by the protein A-antibody bridge and could easily be separated from nonbound cells. The erythrocytes were lysed by distilled H_2O shock and the Ly 5^+ cells were freed and retained for testing. These cells were 82-87% pure as determined by fluorescence testing with specific antisera. The total *in vitro* time for these cell separations was relatively short, probably less than 4 hr.

Figure 3 shows how the isolated cell populations from each step of separation were mixed with RL1 or YAC tumor cells and adoptively transferred to appropriate mice. The *in vitro* manipulated cells were thus returned to an *in vivo* environment.

Table 3 depicts the results of the adoptive transfer conditions. Spleen cells alone, although they contained the NK cells, would not demonstrate protection, probably because of insufficient numbers of NK cells. Not until the Ly 5^+ Thy 1^- cells were highly enriched could protection be seen, but then it was quite remarkable. Further, lysis of this population by Ly 5 antisera showed that the population carrying this surface antigen was definitely involved in protection from the tumor. Such manipulations are impossible in a totally *in vivo* situation.

By such separations, NK cells or other desired populations could be isolated *in vitro*, exposed to chemical treatment to assess direct effects, particularly if *in vitro* metabolic activation could be carried out, and returned to the natural *in vivo* environment in a syngeneic host for testing. Likewise, such tests could be done on cells from chemical-pretreated animals.

Combining *in vitro* manipulation with adoptive transfer and *in vivo* functional analysis is one way in which immunotoxicologic studies might be carried out using currently available methodology and averting possible erroneous conclusions caused by problems associated with *in vitro* survivability.

New Approaches to Screening for Immunotoxicants

Recently, Kutz et al. (*5*) have applied the Mishell-Dutton *in vitro* antibody-forming cell system to screening a large number of environmentally relevant compounds. Many of these chemicals were shown to be immunosuppressive but directly cytotoxic to lymphoid cells *in vitro* at suppressive levels (Table 4). These chemicals included the organotins, several heavy metals and polychlorinated biphenyls. Others, shown in Table 5, were suppressive but not cytotoxic. Most notably, azathioprene was suppressive *in vitro* at levels easily attained *in vivo*

Table 4. Effects of various chemicals on the direct PFC response and viability of Mishell-Dutton cultures.

Compound	90% PFCSD, μg/culture (mean ± SD)[a]	50% VRD, μg/culture (mean ± SD)[a]
Cytotoxic		
DBTC	0.092 ± 0.003	0.06 ± 01
DPTC	0.46 ± 0.006	0.27 ± 06
DOTC	3.80 ± 0.15	1.18 ± 05
DETC	4.77 ± 0.16	3.72 ± 06
DMTC	5.21 ± 0.35	3.53 ± 03
Propyl gallate	8.95 ± 0.13	5.08 ± 36
Mercuric chloride	8.77 ± 0.35	8.11 ± 14
Cadmium chloride	8.90 ± 0.24	9.43 ± 42
Aroclor 1254	17.25 ± 1.60	17.92 ± 40
Aroclor 1248	19.02 ± 0.33	17.72 ± 19
Nickel chloride	45.42 ± 0.09	37.93 ± 80

[a] 90% PFCSD = 90% plaque-forming cell suppressive dose compared to nontreated positive controls; 50% VRD = 50% viability reducing dose. Data are expressed as means ± standard deviations of triplicate cultures (*5*).

Table 5. Effects of various chemicals on the direct PFC response and viability of Mishell-Dutton cultures.

Compound	90% PFCSD, μg/culture (mean ± SD)^a	50% VRD, μg/culture (mean ± SD)^a
Immunosuppressive (at noncytotoxic doses)		
Azathioprine (Imuran)	0.51 ± 0.19	16.39 ± 0.52
Gallic acid	7.56 ± 0.07	< 300
Methyl paraben	98.90 ± 0.95	265.36 ± 11.08
Dextran sulfate	101.17 ± 5.85	< 300
Vanillin	199.75 ± 6.64	< 300
No detectable effect		
Chromic chloride	< 300	< 300
Cyclophosphamide	< 300	< 300
Lead chloride	< 300	< 300
Sodium chloride (182 μg Cl)	< 300	< 300

and with similar suppression kinetics. Thus, some degree of predictability was achieved with this system. Cyclophosphamide also had no effect on this system, owing to the lack of the required microsomal activation of this chemical. The need for an *in vitro* activation step in this potentially useful procedure is discussed by Tucker (*6*). Vos (*7*) and Boorman (*8*) also report on new methods for assessing the effects of chemicals on the adult immune system and the developing immune system, respectively.

Conclusion

Immunology as related to toxicology is an exciting field of research. We are currently learning which methods are applicable to toxicity testing and improving and adapting others to make them suitable. While no one magic test will suffice, it may be possible in the very near future to report the development of more rapid, reliable, economical screening methods for a wide range of immune functions which have predictive value to the whole animal.

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Environmental Health Perspectives
Vol. 43, pp. 115-121, 1982

Use of the Enzyme-Linked Immunosorbent Assay (ELISA) in Immunotoxicity Testing

by J. G. Vos, E. I. Krajnc* and P. Beekhof*

Based on an earlier described macromethod for the routine measurement of IgM and IgG in rat sera, a mechanized micro enzyme-linked immunosorbent assay (ELISA) was developed. The assay was performed in the wells of microtiter plates thus minimizing the quantities of reagents and antisera needed. Data on reproducibility of the assay and calculation of IgM and IgG levels are provided.

For the functional assessment of the humoral immunity of the rat, ELISA is a powerful tool. In an earlier report, assays for the titration of thymus-independent IgM antibodies to *E. coli* LPS and the IgM and IgG response to the thymus-dependent antigen tetanus toxoid were described. More recently it was shown that the antigen ovalbumin elicits a thymus-dependent IgM, IgG and IgE response which could be readily measured with the enzyme immunoassay, as well as a delayed-type hypersensitivity reaction. As the optimum ovalbumin concentration for both types of reactions was the same, it is concluded that the ovalbumin model offers the advantage that both humoral and cellular immunity can be studied simultaneously in the same animal.

Introduction

In clinical immunology, serum immunoglobulin (Ig) levels are routinely determined parameters. Also in the field of immunotoxicology, determination of main Ig classes may provide valuable information about chemically-induced alterations of the humoral immunity (*1*). A routine method for measurement of serum Ig levels is by single radial immunodiffusion (SRID). An alternative method is the enzyme-linked immunosorbent assay (ELISA). After the introduction of the ELISA for the determination of rabbit IgG (*2*), this technique has now become used in a large and still increasing number of applications which include measurements of antigens, haptens and antibodies (*3,4*).

In an earlier report, we have described a "sandwich" ELISA for the routine measurement of total IgG and IgM levels in rat sera (*5*) using a mechanized macro-ELISA system (*6*). In the "sandwich" ELISA, antigen (e.g., rat IgM) reacts with excess solid-phase antibody. After washing, the bound antigen is reacted with excess enzyme-labeled antibody. After further washing, the bound label is assayed, providing a direct measure of the amount of antigen present. IgM and IgG measurements by ELISA correlate well with results obtained with SRID in sera of rats of various age groups. In addition, the precision of the enzyme immunoassay was the same as that obtained with the SRID. However in comparison with the latter method, the ELISA was less time-consuming and the amount of antiserum needed was one order of magnitude lower, and two orders of magnitude lower in the mechanized micro-ELISA that will be described here.

For the functional analysis of the humoral immunity, ELISA is also a powerful tool. In the ELISA for antibodies, antigen is coupled to a solid phase, incubated with the test serum, and the amount of antibody bound to the solid phase is quantified by

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peroxidase-labeled class-specific anti-immunoglobulins. In an earlier report (*5*), ELISA and the passive hemagglutination test were compared in the titration of rat antibodies against *E. coli* lipopolysaccharide (LPS) and tetanus toxoid. Generally ELISA was more sensitive than the hemagglutination reaction and has the major advantage that, by using class-specific enzyme-labeled antisera, both IgM and IgG antibodies could be measured. Based on the similarity of the primary and secondary immune response to LPS, suggesting that immunologic memory did not develop after primary immunization (*5*), and the similarity of the IgM response to *E. coli* LPS in athymic nude rats versus thymus-bearing normal littermates (*7*), it is evident that the humoral response of the rat to *E. coli* LPS is a thymus-independent phenomenon. This means that so-called helper T-cells are not involved in the generation of an immune response. Based on the rapid acceleration of the synthesis of IgG antibodies upon secondary immunization with tetanus toxoid (*5*) and the absence of IgM and IgG antibody responses in athymic nude rats (*7*), tetanus toxoid represents a thymus-dependent antigen.

In the present report, a mechanized micro-ELISA is described for the routine measurement in toxicity studies of rat IgM and IgG, and further improvements and data on reproducibility and calculation of IgM and IgG levels are given. In addition, results of a recent study (*8*) are discussed showing that the antigen ovalbumin elicits a thymus-dependent IgM, IgG and IgE response as well as a delayed-type hypersensitivity response. As the optimal antigen dose for the humoral and cell-mediated reactions is the same, this model offers the advantage that both types of function tests can be studied simultaneously in the same animal.

Antisera and Conjugates

Goat-anti-rat IgM (GaRaIgM) was obtained commercially from Nordic Pharmaceuticals and Diagnostics (Tilburg, The Netherlands). Anti-rat IgG was prepared by hyperimmunization of a sheep with purified IgG emulsified in complete Freund's adjuvant (Difco). The antiserum to IgG (SaRaIgG) was rendered monospecific by absorption with $F(ab')_2$ fragments, obtained by peptic digestion of rat IgG. Anti-rat IgE was prepared by hyperimmunization of a sheep with IgE emulsified in complete Freund's adjuvant. Rat IgE was isolated by salt fractionation, gel filtration and ion-exchange chromatography from serum of Lou rats bearing plasmacytoma IR 162. The antiserum (SaRaIgE) was absorbed with normal rat and rat IgA myeloma (IR 22) serum and fractionated over Sephadex G-200 (Pharmacia, Uppsala, Sweden) to isolate the 7S fraction devoid of immune complexes and most of the excess of rat serum proteins. Immunoglobulin fractions of the three antisera were prepared by caprylic acid factionation (*9*).

The antisera against IgM, IgG and IgE appeared to be monospecific as tested by gel precipitation (Ouchterlony technique). In addition, GaRaIgM and SaRaIgG were tested for specificity in the more sensitive ELISA. For that purpose, wells of polystyrene microplates were coated with GaRaIgM or SaRaIgG, incubated with serially diluted normal rat serum, and finally incubated with peroxidase-labelled SaRaIgG and GaRaIgM. In this test system, the GaRaIgM appeared to contain less than 1% cross-reacting (anti-IgG) antibodies, whereas anti-IgM antibodies were virtually absent ($< 0.01\%$) in the SaRaIgG. A similar specificity (less than 0.01% cross-reacting anti-IgG antibodies) could be demonstrated for SaRaIgE when the heat lability of rat IgE antibodies was utilized. When peroxidase-labelled SaRaIgE was used, no antibodies were detected in high-titered anti-OA sera (^{2}log IgG titers of approximately 15) when the rat sera were heated for 2 hr at 56°C.

Horseradish peroxidase (PO), type VI, R.Z.3, was obtained commercially from Sigma Chemical Co. (St. Louis, USA). PO was conjugated to the antisera by the method of Nakane and Kawaoi (*10*).

Equipment for Micro-ELISA

In the mechanized system (Fig. 1), conventional polystyrene microtiter plates with 12 × 8 flat-bottomed wells were used for through the plate reading of extinction values. A detailed description of the dispenser and simple plate washer is given by Ruitenberg and Brosi (*11*). Extinctions were measured on a commercially available multiple-channel photometer for vertical measurement (Titertek Multiskan, made for Flow Laboratories by Eflab Oy, Helsinki, Finland). Details of the performance of this photometer have been published by Ruitenberg et al. (*12*).

Ruitenberg and Brosi (*11*) also provide commercial sources for equipment for manual dispensing, for an automated washer, and for manual and automated processing (multiple dispenser, washer and photometer).

"Sandwich" Micro-ELISA for Quantification of Rat IgM and IgG

The procedure for the micro-ELISA was largely the same as for the macroassay in which polysty-

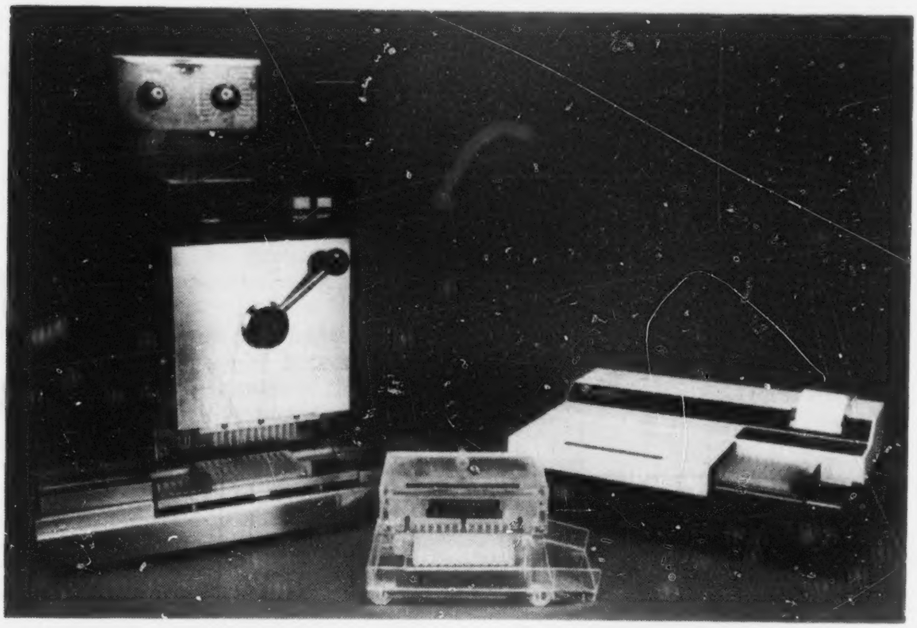

FIGURE 1. Dispenser, plate washer and multichannel photometer for vertical through-the-plate reading of microplates.

rene tubes were used as antigen carriers (*5*). Changes that did improve the test will be discussed below.

Wells of polystyrene microtiter plates were coated by adding 100 μl of anti-immunoglobulin solution (15 μg protein/ml) in sodium carbonate buffer (0.1 M; pH 9.6) with 0.02% NaN_3 as preservative. After incubation at 37°C for 18 hr, in which time the added fluid evaporated, plates were washed by using tap water of neutral pH containing 0.05% Tween 20. For the IgM and IgG analysis sera (reference serum of pooled adult rat serum or pooled serum of control animals as well as the test sera) were diluted with phosphate buffered saline (PBS; 0.01*M*; pH 7.2) containing 2% bovine serum albumin (BSA) to which 0.05% Tween 20 was added. Quantities of 100 μl were added to each well, and plates were incubated under rotation at 37°C for 1 hr. After washing, 100 μl of the conjugate (in an optimal dilution of PBS with 2% BSA and 0.05% Tween 20) was added. Plates were incubated under rotation at 37° C for 2 hr. Excess conjugate was removed by washing. Finally, the amount of enzyme bound to the wells was determined by adding 100 μl of the conventional substrate for ELISA (5-aminosalicylic acid and H_2O_2). Earlier (*5*), the substrate was prepared by dissolution of 80 mg of 5-aminosalicylic acid in 100 ml of distilled water at approximately 70°C; directly prior to use the pH of this solution was brought to 6.0 with 1*N* NaOH. As shown in Figure 2, dissolution of the substrate in 0.05*M* maleate buffer of pH 6.0 markedly improved the slope of the standard curve. Also the concentration of H_2O_2 strongly influenced the assay. Earlier, to 9 ml of the 5-aminosalicylic acid solution 1 ml of 0.05% H_2O_2 was added. As shown in Figure 3, the

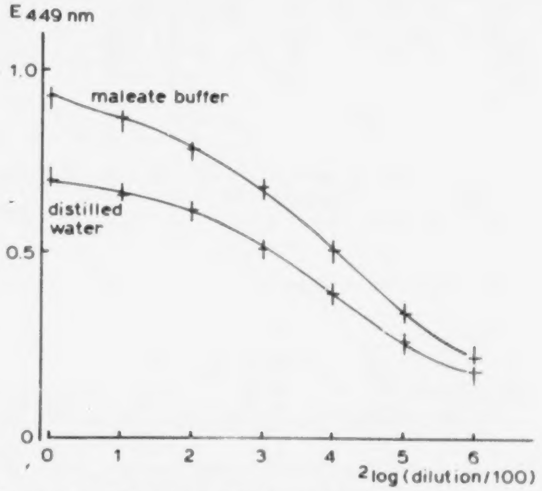

FIGURE 2. Difference in IgM standard curve by dissolution of the substrate 5-aminosalicylic acid in distilled water or in 0.05*M* maleate buffer (pH 6.0).

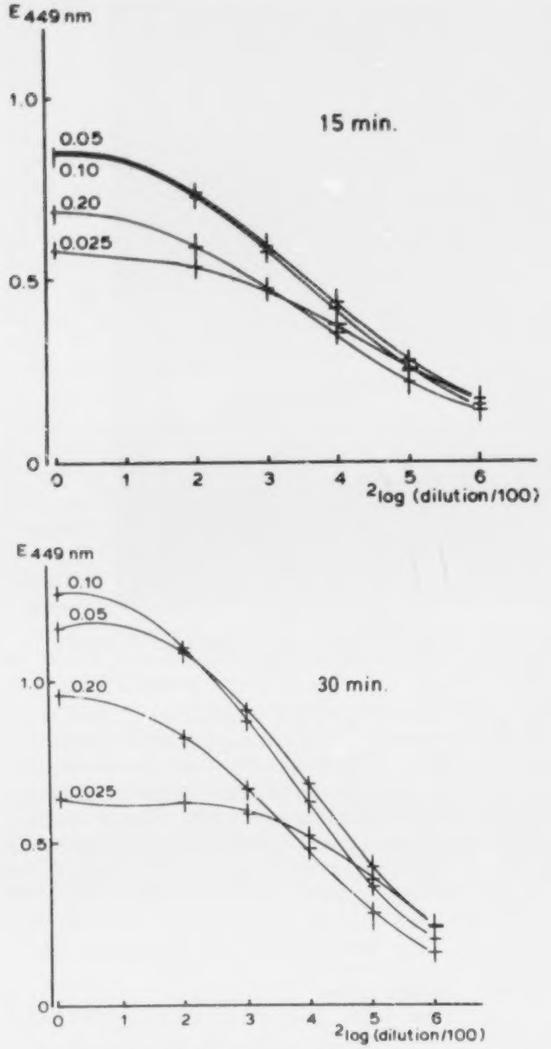

FIGURE 3. Effect of different hydrogen peroxide concentrations (0.025-0.2%) added to the substrate on extinction values in the IgM micro-ELISA: (top) after 15 min; (bottom) after 30 min incubation.

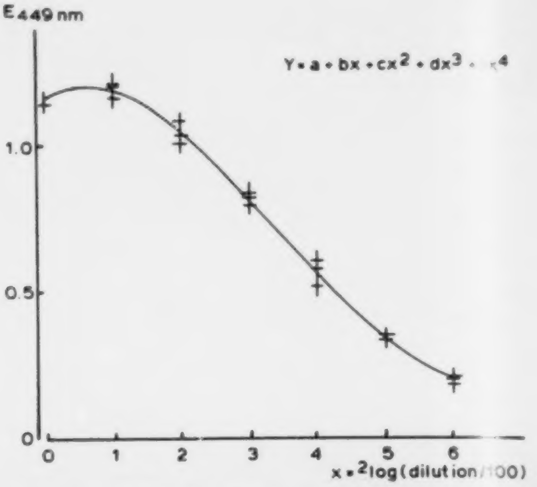

FIGURE 4. Standard curve calculated by least-squares method for measuring rat IgM by micro-ELISA.

concentration of H_2O_2 is critical, as a twofold lower (0.025%) or fourfold higher (0.20%) concentration of H_2O_2 caused a pronounced decrease in extinction values at low serum dilutions, resulting in a standard curve with a flat slope. Since the concentration of H_2O_2 in the stock solution decreases in time, a concentration of 0.10% H_2O_2 was chosen to be added to 5-aminosalicylic acid, and 100 μl amounts of the freshly prepared substrate were added to the wells. After incubation at room temperature for an optimal period of 30 min (Fig. 3), extinctions were measured on the multichannel photometer.

For the quantification of IgM and IgG levels, standard curves consisting of seven twofold serial dilutions of a reference serum or a pooled serum of the control group, were calculated by least-squares fit of the fourth-degree polynomial (Fig. 4). Test sera were analyzed in one dilution. In addition, a conjugate control was included (i.e., anti-immunoglobulin-coated well, to which conjugate was added and after proper incubation and washing, the sub-

Table 1. Statistical analysis for estimating differences in serum IgM levels in control and hexachlorobenzene-treated rats.[a]

Group	IgM level in standard serum, %					Mean	SD	*F* ratio
1	28.2	30.95	48.8	35	24.15	31.4938	7.7271	
	25.7	29.85	39.3					
2	31.25	30.95	27.45	26.45	34.15	34.1125	9.1273	1.3952
	52.85	27.5	42.3					
3	33.9	54	39.05	63.75	54.15	47.5313	13.5300[b]	3.0659[c]
	44.45	27.35	63.6					

[a] IgM HCB PO 78/70.

[b] Significance by Student *t*-test: $p < 0.05$.

[c] Significance by Welch *t*-test: $p < 0.05$.

strate). In order to eliminate variations between plates, a standard curve was included in each plate. This means that per microtiter plate, 21 wells were used for the standard curve, 3 for the conjugate control, and 72 for the test samples (triplicate analyses of 24 rats). Differences in group mean values were estimated by Student's t-test, and results expressed either as percentage of the IgM or IgG level in the standard serum (Table 1) or as percentage of the control serum. The calculations were performed on a BASIC programmable topdesk computer.

The reproducibility of the enzyme immunoassay for IgM and IgG was determined by comparing extinction values of twofold dilutions of the reference serum, determined in five sessions spread over a 4-month period. As shown in Figure 5, extinction values determined on different days were nearly identical for both IgM and IgG, demonstrating the excellent reproducibility of the assays. The data shown in Figure 5 were obtained by macro-ELISA. Preliminary data of the micro-ELISA however are indicative of a high reproducibility as well.

A problem encountered in the use of micro-ELISA which may influence the results lies in the position of the wells in the microtiter plates. As shown in Figure 6, the wells at the edge of the microplates showed higher extinctions, and wells in the center showed lower extinction values. This difference may originate from inhomogenicity in the polymerization process of the polystyrene plate, which in turn may influence the coating efficiency. Effects of well position however are also known from lymphocyte transformation studies in microplates in which no coating is involved. The systematic influences of well position on the outcome of the test is reduced by an at random distribution of the triplicates of each sample. However, the variation due to well position is small when compared to the variation in serum IgM and IgG levels between individual animals. This is demonstrated by the finding that IgG levels of a group of random-bred rats of the Wistar strain, bred and housed under SPF conditions, showed a marked variation (Fig. 7). Also shown in this figure is the dramatic effect of a virus infection on the serum IgG level when rats bred under SPF conditions were housed under conventional conditions after weaning. For example, serum IgG levels of one group housed under conventional conditions and all serological positive for pneumonia virus of mice, were 150% increased in comparison with SPF littermates. Besides the induction of specific antibodies, which may markedly increase Ig levels, the microbiological status of experimental animals is extremely important in immunotoxicity testing as various infectious agents

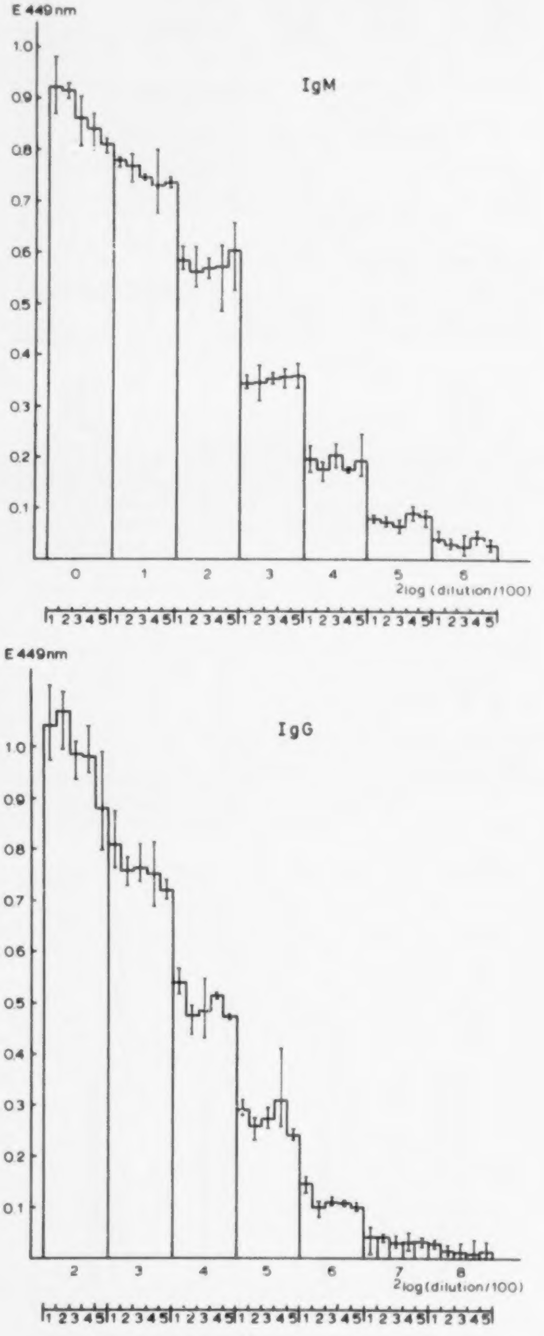

FIGURE 5. Reproducibility of extinctions of twofold serial dilutions of the standard serum as measured with the macro-ELISA. Analyses of (*a*) IgM and (*b*) IgG were performed in five sessions spread over a 4-month period. Results of triplicate analyses are expressed as mean and extreme values.

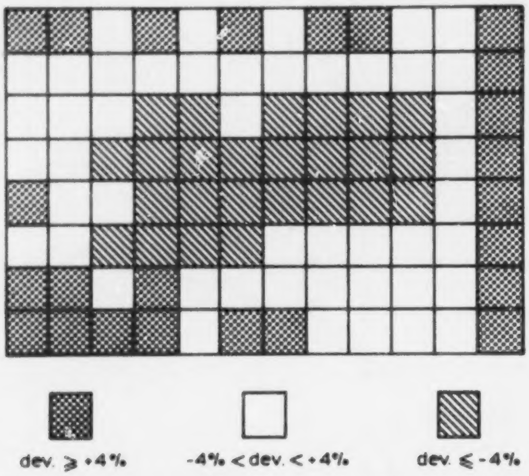

FIGURE 6. Effect of well position in microplate on extinction values of rat serum tested in one dilution. Results of IgM measurements are given as a percentage of the deviation of the mean.

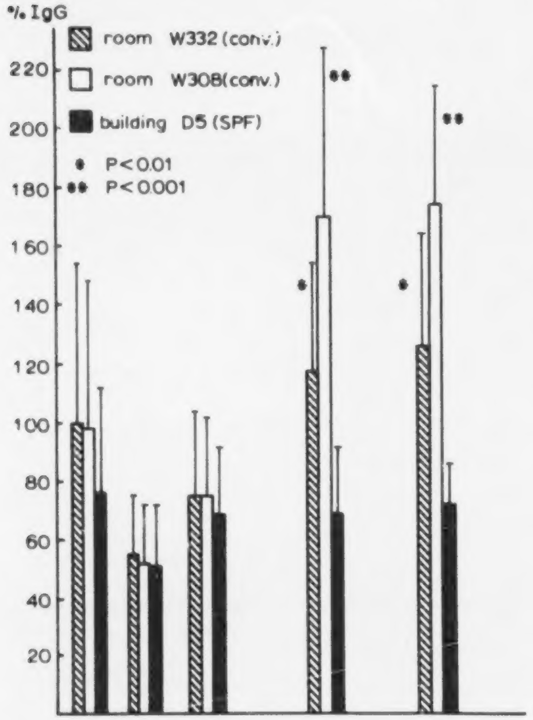

FIGURE 7. Serum IgG levels of rats bred under SPF conditions, and after weaning under conventional or SPF conditions (means ± SD of 9-10 rats per group). Note the large variation of IgG levels in SPF animals and the strong increase in IgG levels of rats housed under conventional conditions and infected with pneumonia virus of mice (*13*).

do modify the immunological responsiveness of the host (*14*). The outcome of an experiment may be influenced by such an infection, particularly when function tests of the immune system are carried out.

ELISA for Determining IgM, IgG and IgE Titers to Ovalbumin

The immunization procedure and the micro-ELISA are described in detail elsewhere ([illegible]). In short, rats are immunized by a subcutaneous injection in the hind footpads with an optimal dose of 100 μg ovalbumin (crystallized five times; Serva Feinbiochemica, Heidelberg, Germany) dissolved in 0.05 ml PBS and emulsified in 0.05 ml of complete H37Ra adjuvant (Difco Laboratories, Detroit, Michigan). For the determination of anti-ovalbumin antibodies routinely day 10 and 22 sera are used.

The enzyme immunoassay was performed with the dispenser and washer shown in Figure 1. Polystyrene microplates were coated with 5 μg ovalbumin/ml sodium carbonate buffer, 0.1*M*, pH 9.6. After coating for 2 hr at 37°C, the microplates were washed with tap water of neutral pH containing 0.05% Tween 20. Serial twofold dilutions of the rat sera were prepared in PBS (0.01*M*, pH 7.2) containing 0.05% Tween 20. Since a cross-reaction was observed between antibodies against OA and bovine serum albumin, the latter protein was not added to the serum diluent or to the conjugate. Microplates containing 100 μl of the diluted sera were incubated at 37°C for 1 hr. After being washed, 100 μl of conjugate was added after proper dilution with PBS (0.01*M*, pH 7.2) containing 0.05% Tween 20. Microplates were incubated under rotation at 37°C for 2 hr. The trays were washed again, and 100 μl of substrate (5-aminosalicylic acid and H_2O_2) was added. After incubation at room temperature for approximately 1 hr, the reaction product was evaluated visually and expressed as ^{2}log of the highest dilution giving a positive color reaction.

As shown in Figure 8, the optimal antigen dose for functional assessment of the IgM, IgG and IgE antibody response lies around 100 μg ovalbumin. It also appears that the same concentration is optimal for the delayed-type hypersensitivity (DTH) reaction as parameter of the cell-mediated immunity. Therefore, these function tests can be combined in the rat, which decreases the number of experimental animals needed. The absolute thymus dependency of the humoral response to ovalbumin is clearly demonstrated by the negative ELISA IgM and IgG reactions in 1:2 diluted sera of athymic

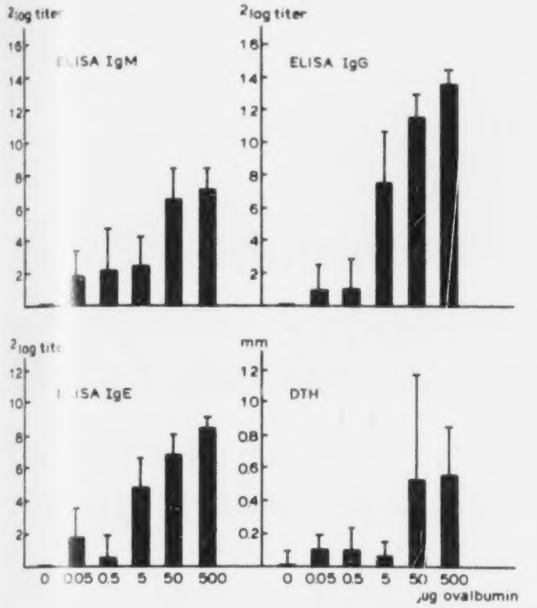

FIGURE 8. ELISA IgM, IgG and IgE titers and delayed-type hypersensitivity (DTH) reactions as measured 3 weeks after SC immunization of Wistar rats with various concentrations of ovalbumin, emulsified in H37Ra adjuvant. DTH reactions are expressed as differences in thickness of 24 hr skin reaction between ovalbumin-challenged and control ear (*8*).

rats, whereas thymus-bearing littermates had a high response (serum dilutions of 1:30,000 still yielded a positive IgG reaction) (*7*).

Instead of endpoint titration, IgM, IgG and IgE antibodies to ovalbumin can also be quantified by measurement of extinction values in twofold serially diluted sera. As shown elsewhere (*8*), at certain dilutions of the antisera to ovalbumin, extinctions were measured that approached a linear function when plotted against the dilution, while within the IgM, IgG or IgE assays, the slopes of the linear area were the same. The same holds true for the measurement of IgM antibodies against *E. coli* LPS (*5*). By such an approach, the discriminatory power of the enzyme immunoassay increases which may be of importance when subtile effects on the humoral immunity are studied.

Like all immunological tests in which anti-immunoglobulins are used, the specificity of the enzyme immunoassay largely depends on the quality of the antisera and conjugates used. Conventional commercial antisera frequently do not meet the high standard needed. However, it can be expected that in the near future high quality monoclonal antisera, obtained by the hybridoma technique, will be commercially available. Because of simplicity and sensitivity, coupled to a very economic use of antisera, it can be expected that also in the toxicology area enzyme immunoassays will become routine methods to quantify antigens and antibodies. There is the added advantage that the (enzyme-labeled) antisera can also be used in histology, e.g., for the immunocytochemical demonstration of plasma cells.

The authors thank Dr. J. Nagel and Dr. A. M. Hagenaars of the Laboratory for Immunochemistry for the preparation and conjugation of the antisera.

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Environmental Health Perspectives
Vol. 43, pp. 123-127, 1982

In Vitro Immunotoxicological Assays for Detection of Compounds Requiring Metabolic Activation

by Anne N. Tucker,* Virginia M. Sanders,* Patricia Hallett,* Bernadine M. Kauffmann* and Albert E. Munson*

A system for metabolic activation of cyclophosphamide (CP), consisting of a crude microsomal fraction of mouse liver and necessary cofactors (S9 mix), was interfaced with three murine cell culture assays for immunotoxicity. These assays were: the Mishell-Dutton assay for *in vitro* antibody formation, splenic lymphocyte responsiveness to mitogens and bone marrow cell cultures. There was no effect of CP at doses up to 261 μg/ml (1*mM*) on any of the parameters measured unless S9 mix was included. Much greater potency was achieved if the S9 mix was prepared from livers of mice pretreated with phenobarbital. Under these conditions a dose-related inhibition of plaque-forming cells (PFC) in the Mishell-Dutton assay was observed, yielding an ED_{50} of 6.3 μg/ml. When splenic lymphocytes were exposed to CP in the presence of induced S9 mix, a dose related inhibition of the response to the B-cell mitogen, lipopolysaccharide (LPS), and to the T-cell mitogen, concanavalin A (Con A), was observed. For the optimum LPS concentration, the ED_{50} for CP was 8.1 μg/ml; for the optimum concentration of Con A, the ED_{50} was 6.7 μg/ml. DNA synthesis was not inhibited by the doses used. When bone marrow cells were exposed to CP in the presence of induced S9 mix, the stem cell population, enumerated by colonization in semisolid medium, was reduced in a dose-dependent manner, with an ED_{50} of 5.2 μg/ml. Again, DNA synthesis was not affected unless higher doses of CP were used.

In vitro assays for defining toxicological effects are becoming increasingly important as the burden of necessary investigations increases. Our laboratory has successfully interfaced a system for metabolic activation with several *in vitro* assays for immunotoxicity, thus widening the scope of these assays to include immunosuppressive agents which are not direct acting. Cyclophosphamide (CP) was chosen as a positive control in these assays since it requires metabolic activation (*1*) and is immunosuppressive in the mouse (*2*).

Metabolic Activation System

A crude microsomal fraction was prepared from livers of male BALB/c mice to serve as a source of enzymes for metabolic activation. In some cases, the enzymes were induced with phenobarbital by placing 1 mg/ml in the animals' drinking water for 1 week until 24 hr prior to sacrifice by cervical dislocation. Livers were removed aseptically and homogenized in 3 ml of cold, sterile 1.15% KCl/g liver. This homogenate was centrifuged at 9000*g* and the supernatant (S9 fraction) removed and frozen in 1 ml aliquots. When used, a tube of S9 was removed from storage at –70°C, thawed and added to a solution of cofactors. This S9 mix contained 3 mg S9 protein, 2 *mM* NADP, and 35 *mM* isocitrate in sterile culture medium and was added directly to cell suspensions, 0.1 ml/ml of cell suspension.

Mishell-Dutton Assay

The Mishell-Dutton *in vitro* antibody-producing assay has been found useful for recognizing immu-

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Table 1. *In vitro* inhibition of the primary antibody response to sheep erythrocytes by cyclophosphamide in the Mishell-Dutton assay.[a]

Cyclophosphamide					
mM	μg/ml	S9 Mix	PFC/culture	10^6 Cells	PFC/10^6 cells
None	0	None	1760 ± 210	5.55 ± 0.47	31
1.0	261	None	1707 ± 154	5.27 ± 0.17	32
None	0	Naive	2520 ± 218	5.00 ± 0.23	50
0.1	26	Naive	1807 ± 140	4.12 ± 0.59	43
0.3	78	Naive	1035 ± 121	5.02 ± 0.81	20
1.0	261	Naive	727 ± 144	2.92 ± 0.34	24
None	0	Induced	2227 ± 154	5.05 ± 0.41	44
0.01	2.6	Induced	1835 ± 161	3.62 ± 0.53	50
0.03	7.8	Induced	795 ± 111	3.65 ± 0.18	21
0.10	26	Induced	None	1.88 ± 0.23	[illegible]

[a] Spleens were obtained from male BALB/c mice, 10 weeks of age. Plaque forming cells (PFC) were enumerated on day 5 culture and are expressed as the mean ± standard error (n = 3). Background (no antigen) was 102 ± 23 PFC/culture.

nosuppressive chemicals (*3*). In addition to specificity, the system offers the advantage of complex cellular interactions involved to achieve the ultimate response. The disadvantage of the assay, as pointed out by Kutz et al. (*4*), is that chemicals which require activation to reactive metabolites are not detectable. With the modification described here, chemicals which require metabolic activation can be tested in the assay.

The modified assay was carried out by first preparing a spleen cell suspension in RPMI 1640 culture medium and adjusting the cell concentration to 1.5 × 10^7 cells/ml. The suspension was then distributed into 10 × 35 mm plastic petri plates, 1 ml per plate. To each plate was added 0.1 ml of S9 mix and CP (Sigma) as indicated in Table 1. The plates were incubated at 37°C for 1 hr in a culture box gassed with a mixture of 10% CO_2, 7% O_2, 83% N_2 on a rocker platform.

As described elsewhere (*5*), the 1 hr preincubation was necessary because of loss of response of the assay when S9 mix was added at the time of antigen and left in for the 5 days of culture. Following this incubation period, the contents of each plate were transfered to a sterile plastic tube and centrifuged at 2000*g* for 10 min. Each pellet was resuspended in 1 ml RPMI 1640 (1 × 10^7 cells/ml), 0.1 ml fetal calf serum and antigen (5 × 10^6 sheep erythrocytes) were added, and 1 ml of the resulting suspension was placed in a fresh petri plate. The plates were then placed in the culture boxes, gassed with the special mixture, and incubated at 37°C with gentle rocking and daily feeding as described by Mishell and Dutton (*6*). On day 5, the contents of each plate were collected for enumeration of plaque-forming cells (PFC) using Cunningham slides (*7*). Cell counts were performed by using a Model ZBI Coulter counter.

The effect of CP on the primary antibody response of splenic lymphocytes to sheep erythrocytes using the modified Mishell-Dutton assay is shown in Table 1. The control value (1760 PFC/culture) can be compared to a mean control value from six experiments not involving the modification of 2680 ± 273. Cyclophosphamide had no effect up to 1*mM* (261 μg/ml) when S9 mix was not included, but produced a dose-dependent inhibition in the presence of S9 from livers of either naive or phenobarbital-induced mice. The use of the induced preparation caused a tenfold increase in potency of CP, as would be expected. The preparations used in this experiment had been stored for 3 months at –70°C. Although the fractions were more active when used shortly after preparation, the induced preparation was slightly inhibitory at that time.

There is an effect on cell yield at the higher doses of CP when S9 mix is included which seems to be related to the reduced yield of PFC/culture. For comparison, PFC/10^6 cells is shown in the last column of Table 1. Approximate ED_{50} values for CP when activated by induced S9 are 6.3 μg/ml with PFC/culture as endpoint and 7.8 μg/ml with PFC/10^6 cells as endpoint.

Splenic Lymphocyte Cultures

We have been using murine splenic lymphocyte cultures to assess immunotoxicity of a variety of chemicals. The parameters measured include effects on DNA synthesis and response of the lymphocytes to mitogens. The use of the S9 mix and effect of CP in this assay will be described.

Cell suspensions were prepared in RPMI 1640 by using spleens from male CD-1 mice, a random-bred albino from Charles River, which were 10-12 weeks of age. This strain is also being used in our laboratory for *in vivo* toxicological studies. Following collection of the cells by centrifugation, the pellet

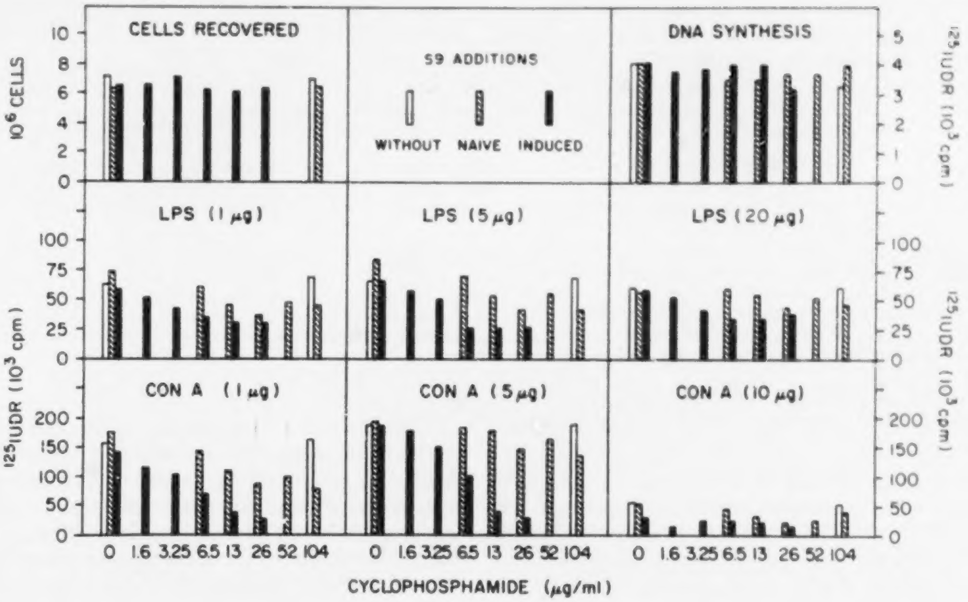

FIGURE 1. Effect of cyclophosphamide on cultured murine splenic lymphocytes. Suspensions of lymphocytes were exposed for 1 hr to cyclophosphamide at the doses indicated. In some cases S9 mix from either naive or phenobarbital induced mouse livers was included, as indicated by hatched or solid bars, respectively. The values shown are means from triplicate exposures.

was resuspended in RPMI 1640 containing 10% calf serum and adjusted to 5 × 10^6 cells/ml. Cyclophosphamide and S9 mix were added to triplicate siliconized glass tubes, each containing 5 ml of cells. A 1-hr incubation was performed with the tubes in a roller drum in a 37°C, 5% CO_2 incubator. At the end of this incubation, the cells were collected by centrifugation and resuspended in RPMI 1640 containing 10% fetal calf serum for counting. Viability was determined by trypan blue exclusion.

For determination of DNA synthesis, a portion of the cell suspension was adjusted to 3 × 10^6 cells/ml and incorporation of ^{125}I-iododeoxyuridine (IUdR) was measured in the presence of 1 µ*M* fluorodeoxyuridine (FUdR). In this assay, 6 replicate 200 µl aliquots were incubated with label in a microtiter plate for 1 hr, and the cells were collected with a Titer k Cell Harvester. Quantitation of ^{125}I was by gamma counter.

The response of splenic lymphocytes to the B-cell mitogen, lipopolysaccharide (LPS) from Salmonella, and the T-cell mitogen, concanavalin A (Con A), was determined by IUdR incorporation also. Suspensions were adjusted to 5 × 10^6 cells/ml and six 100 µl replicates incubated with mitogen in microtiter plates in a 37°C, 10% CO_2 incubator for 48 hr. Three concentrations of each mitogen were used. The samples were then pulsed for 18 hr with ^{125}IUdR/FUdR and the ^{125}I incorporation determined as described for DNA synthesis.

Table 2. DNA synthesis in spleen cells exposed to cyclophosphamide in the presence of S9 mixes.

CP, µg/ml	^{125}IUdR incorporation, cpm/6 × 10^5 cells[a]		
	Without S9 Mix	Naive S9 Mix	Induced S9 Mix
0	4093 ± 128	4109 ± 90	4089 ± 273
1.6	ND	ND	3791 ± 256
3.25	ND	ND	3897 ± 75
6.5	ND	3515 ± 236	4026 ± 200
13	ND	3460 ± 194	4020 ± 201
26	ND	3742 ± 203	3160 ± 76
52	ND	3680 ± 101	ND
104	3607 ± 600	4045 ± 214	ND

[a] Means ± standard error (n = 3); ND = not done.

Cell recovery was not affected by CP, even when S9 mix was included. This is illustrated graphically in Figure 1. Viability, which was 91% in the control was also not changed by any of the treatments. DNA synthesis was also unaffected, as shown in Table 2 and Figure 1. Data from other experiments which are not shown here indicate that CP in the presence of induced S9 inhibits DNA synthesis at higher doses than used in the experiment described.

Mitogenicity data are shown in Table 3 and Fig-

Table 3. Spleen cell response to mitogens in the presence of cyclophosphamide (CP) or S9.

Mitogen	^{125}IUdR incorporation, 10^3 cpm/5 × 10^5 cells[a] Without S9, no CP	Without S9, CP = 104 μg/ml	Naive S9, no CP	Induced no C
LPS				
1 μg/well	63.7 ± 3.3	69.8 ± 2.9	73.3 ± 0.3	67.7 ±
5 μg/well	66.0 ± 3.6	68.9 ± 0.8	85.2 ± 7.8	67.4 ±
20 μg/well	60.3 ± 4.3	61.0 ± 1.8	63.7 ± 2.9	58.9 ±
Con A				
1 μg/well	155.9 ± 5.4	161.1 ± 2.6	173.1 ± 2.2	138.3 ±
5 μg/well	188.4 ± 2.7	188.4 ± 2.7	198.8 ± 4.9	181.0 ±
10 μg/well	55.4 ± 3.2	51.1 ± 3.8	51.1 ± 3.2	27.7 ±
None	1.5 ± 0.2	1.9 ± 0.2	1.7 ± 0.1	2.1 ±

[a]Mean ± standard error (n = 3).

ure 1. Table 3 gives the control values, and Figure 1 is a graphic representation of mitogen responsiveness when both CP and S9 are included in the 1-hr preincubation. A dose-dependent inhibition by CP is observed when S9 is included, and this inhibition occurs at lower doses if S9 from phenobarbital induced animals is used as the source of activating enzymes. The ED_{50} in this case is 8.1 μg/ml for LPS (5 μg/well) and 6.7 μg/ml for Con A (5 μg/well).

Bone Marrow Cultures

Immediately after removal of spleens from CD-1 mice, the hind limbs were removed and placed on ice. Using aseptic techniques, the femurs were trimmed and the contents flushed into a plastic centrifuge tube with α MEM (minimum essential medium) containing 10% fetal calf serum and adjusted to 3 × 10^6 cells/ml. Aliquots of 5 ml were placed in siliconized glass tubes, S9 mix and CP added where indicated, and the tubes incubated 1 hr in the roller drum as described for splenic lymphocytes. Cells were then collected by centrifugation and resuspended in α MEM with 10% fetal calf serum. Cell concentration and viability were determined, then the cells were adjusted to 3 × 10^6 cells/ml for determination of DNA synthesis as described for lymphocytes.

Viable stem cells were then enumerated by colonization in semisolid medium. For this determination, the cells were adjusted to 10^5 cells/ml in α MEM-containing 10% fetal calf serum, 5% horse serum, 1.8% methyl cellulose, and 10% L-cell conditioned medium. A 2-ml portion of suspension was placed in each of three wells in a six-well Linbro plate, and the plates incubated at 37°C, 10% CO_2, 95% humidity for 7-10 days. Colonies, defined as clusters of 25 cells or more, were then counted.

The results of a typical dose response experiment using S9 from both naive and phenobarbital induced animals are given in Table 4. Cell yield was not affected by the treatment; viability was 91-93% in controls as well as all high dose samples. As shown in Table 4, there was no inhibition of DNA synthesis at any dose of CP, even when S9 was included. Colony formation was affected; however, in a dose related manner when S9 from either naive animals or phenobarbital induced animals was included. Approximate ED_{50} values for the CP effect on stem cells are 20.8 μg/ml for uninduced S9 and 5.2 μg/ml for induced S9.

Discussion

In vitro activation of CP was first described in Ames' Salmonella microsome test for mutagenicity

Table 4. Effect of cyclophosphamide on DNA synthesis and stem cells in bone marrow cell cultures.[a]

	DNA Synthesis, 10^3 cpm ^{125}I			Stem cell enumeration, colonies/10^5 cells		
CP, μg/ml	Without S9	Naive S9	Induced S9	Without S9	Naive S9	Induced S9
None	33.5 ± 4.0	24.6 ± 3.1	23.2 ± 0.9	163.4 ± 1.0	163.4 ± 0.9	161.1 ± 0.9
3.25	ND	ND	27.0 ± 2.2	ND	ND	104.1 ± 0.7
6.5	ND	24.3 ± 1.3	31.3 ± 2.0	ND	142.4 ± 1.0	66.9 ± 1.5
13	ND	25.7 ± 1.1	33.2 ± 2.5	ND	123.7 ± 1.5	29.3 ± 2.2
26	40.6 ± 3.9	33.7 ± 2.1	ND	162.7 ± 2.2	51.6 ± 2.0	ND

[a]All values represent the mean ± standard error (n = 3). ND = not done.

Table 5. Summary of ED_{50} values for cyclophosphamide activated *in vitro* by S9 mix from phenobarbital-induced mice.[a]

Assay	End point	ED_{50}, μg/ml
Mishell-Dutton	PFC/culture	6.3
	PFC/10^6 cells	7.8
Spleen lymphocyte	Response to LPS (5 μg/well)	8.1
	Response to Con A (5 μg/well	6.7
	DNA synthesis	>26
Bone marrow	Stem cell	5.2
	DNA synthesis	>26

[a] The values shown here were obtained from log dose plots of data shown in the other tables.

(*8*). Since then, it has been used as a model compound in other, more sophisticated mammalian *in vitro* assays. Stetka and Wolff were able to induce sister chromatid exchanges in cultured Chinese hamster ovary cells by including rat liver microsomes with CP (*9*). Recently, an *in vitro* teratogenic assay was described in which CP induced defects when an hepatic microsomal fraction was included (*10*). The present report extends use of *in vitro* activation of CP into the area of immunotoxicology.

A system for metabolic activation of CP *in vitro* has been successfully interfaced with three cell culture assays for immunotoxicity. A summary of the effects of CP when activated by an S9 mix from phenobarbital induced animals is given in Table 5. The approximate ED_{50} values for specific end points in the three assays are very similar, ranging from 5.2 to 8.1 μg/ml or 2.0 to 3.1 × $10^{-5}M$. It is important to note that DNA synthesis is inhibited only when much higher doses of CP are used. The utility of the described assays is tremendously increased by inclusion of the S9 mix, in that they can now be used for screening for immunotoxicity, as well as elucidation of mechanisms of action. As with all *in vitro* assays, it is important to keep the information in context with *in vivo* effects.

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Environmental Health Perspectives
Vol. 43, pp. 129-135, 1982

Assessment of Myelotoxicity Caused by Environmental Chemicals

by G. A. Boorman,*† M. I. Luster,* J. H. Dean* and M. L. Campbell*

Potential antineoplastic agents must be screened for the delayed toxicity that occurs in many cases of drug-induced bone marrow aplasia. *In vitro* clonal assays for hematopoietic progenitor cells have been developed to assess the degree of myelotoxicity. This adverse side effect is often the limiting factor in the development of new cancer chemotherapeutics. In addition, many environmental chemicals are cytotoxic to rapidly proliferating cells, but a systematic assessment of their myelotoxicity has not been performed. We have used clonal marrow assays to investigate a panel of chemicals including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, polybrominated biphenyls, diethylstilbestrol, benzo(a)pyrene and indomethacin. All were immunotoxic, some to pleuripotent hemopoetic stem cells and others to granulocyte-macrophage progenitors, and at concentrations below those causing other toxic manifestations. This shows that these bone marrow clonal assays, and hopefully future ones for erythroid, B- and T-lymphocytes, and megakaryocytes, will provide the specificity and sensitivity necessary to delineate the myelotoxicity of a broad spectrum of environmental chemicals.

Introduction

As one of the most rapidly renewing cell populations, hemopoietic cells have proven very sensitive to cytotoxic agents (*1-5*). Bone marrow failure is a significant complication of cancer chemotherapy and has also been implicated as a result of exposure to numerous drugs (*6-9*) and environmental agents (*10-12*). During the past decade, a variety of semisolid *in vitro* culture techniques have been developed for most of the hemopoietic cell lines. Examination of colony formation of the hemopoietic cells following exposure to various agents has proven to be a sensitive indicator of toxicity as well as a means for mechanistic study of the toxicity of various drugs (*9*).

Murine pleuripotent hemopoietic stem cells can be detected by injecting bone marrow cells into irradiated recipients and counting the number of colonies that form in 8 days (*13*). More differentiated committed progenitor cells can be induced to form colonies in semisolid media by adding appropriate stimulating factors. Currently, clonal assay procedures exist (Fig. 1) for B- and T-lymphocytes, macrophage-granulocyte progenitors, megakaryocytes, eosinophils and erythroid precursors (*14,15*). Colonies arise from proliferation of individual cells and the number of colony forming cells are altered under abnormal physiological conditions following treatment with certain drugs, and in certain disease states such as chronic myelogenous leukemia and polycythemia rubra vera (*15*).

Hemopoietic stem cells appear to have a limited capacity for division. This can be demonstrated by serial passage of bone marrow cells into irradiated recipients (*16*). Serial transfer of the donor cells will permit the marrow tissue to survive beyond the normal life expectancy and shows that bone mar-

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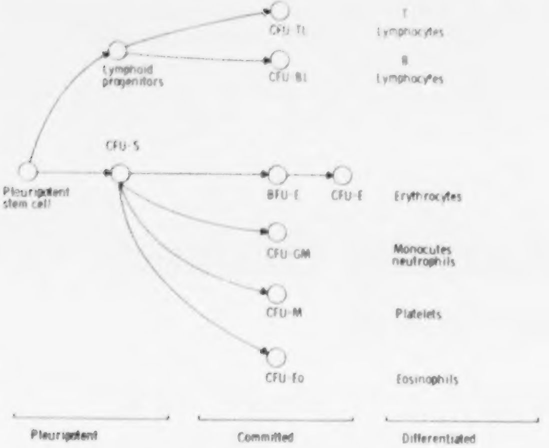

FIGURE 1. Model of hematopoiesis as determined by *in vitro* and *in vivo* clonal assays. CFU-S, colony-forming unit-spleen; CFU-TL and CFU-BL, colony-forming unit-T and B-lymphocyte, respectively; BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; CFU-GM, colony-forming unit-granulocyte-macrophage; CFU-M, colony-forming unit-megakaryocyte; CFU-Eo, colony-forming unit-eosinophil.

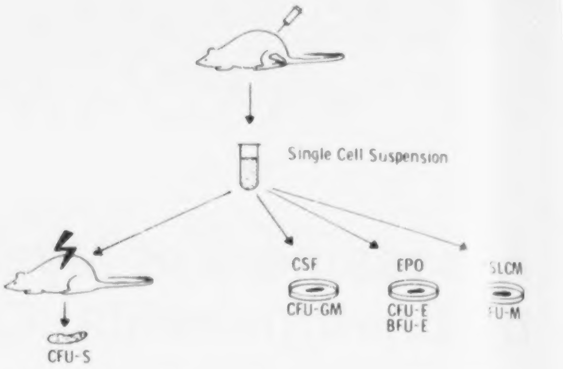

FIGURE 2. Scheme of testing for myelotoxicity. Following exposure to a chemical a single cell suspension is made of femoral marrow. Following enumeration of nucleated marrow cells they are injected into irradiated recipients to measure colony-forming units-spleen (CFU-S) and plated in *in vitro* cultures to which has been added colony-stimulating factor (CSF), erythropoietin (EPO) or pokeweed stimulated lymphocyte culture medium (PSLCM) which will allow the proliferation of granulocyte-macrophage progenitors (CFU-GM), erythroid progenitors (BFU-E and CFU-E) and megakaryocytes (CFU-M), respectively.

row would not be a limiting factor in aging of the mouse. Fetal or embryonic hemopoietic tissue has a greater capacity for serial transfer than from adult mice. However, serial transfer capacity changes very little with aging, suggesting the stem cell compartment is minimally used during adult life (*18*). Several studies have shown that exposure to a toxic agent such as busulfan can induce a permanent stem cell defect that persists throughout the life of the animal without any evidence of recovery (*19,20*). Animals with permanent stem cell defect may have normal bone marrow cellularity and peripheral blood counts, but the residual injury can be demonstrated by increased sensitivity to irradiation (*19*), decreased erythropoietic response following anemia stress (*21*), decreased proliferative capacity of bone marrow or after serial transplantation (*22*) and decreased antibody response (*20*). These animals will eventually die of chronic aplastic marrow failure (*23*).

The concept of residual marrow injury may prove to be very important in toxicology testing. Currently toxicological assessment devotes little effort to understanding myelotoxicity and the usual screening programs would not detect residual stem cell injury since these animals may appear clinically normal. Chronic aplastic marrow failure, frequently a fatal syndrome, has a history of drug or chemical exposure in approximately 50% of the cases (*23*). Although marrow failure is rare, the studies cited above suggest a much larger population may exist with undetected residual stem cell injury following chemical exposure. This population would show decreased ability to respond to situations requiring increased hemopoietic cell production and may be more susceptible to other cytotoxic drugs. While the model of induced stem cell defect and marrow failure was developed in mice, it would appear relevant to man, since it has been shown that patients exposed to chemotherapy show a prolonged decrease in bone marrow colony-forming cells in spite of normal peripheral blood counts, and it has been suggested that the marrow is more fragile in these patients (*24*). Based on the mouse model, these patients would be expected to have less bone marrow reserve when extra hematopoietic cells are needed (Fig. 2).

Assessment of Bone Marrow Capacity by Use of Clonal Assays

Pleuripotent Stem Cells (CFU-S)

Till and McCulloch (*13*) showed that when mouse marrow cells were injected into heavily irradiated recipients, discrete nodules of hemopoietic cells would form in the spleen of the recipients in 8 or 9 days. The nodules, called spleen colonies, contained pure or mixed populations of various hemopoietic cell lines (*15*) and were shown by chromosomal markers (*25*) to arise from individual cells which have been called colony-forming unit-spleen (CFU-S), or pluripotent stem cells.

When isolated, made into a suspension and reinjected into an irradiated recipient, a stem cell colony containing cells of one hemopoietic cell line would give rise to new colonies of all cell lines, demonstrating both the self-renewal capacity and pleuripotentiality of the CFU-S. The relationship of the CFU-S to lymphocytes, an area of controversy, now appears to be resolved with the CFU-S and lymphoid progenitors being derived from a common, more primitive, stem cell (*15,26*). Most CFU-S have a long generation time or remain longer in the G_0 cell cycle phase. When marrow depletion occurs, CFU-S respond by more rapid proliferation and differentiation. As mentioned earlier, CFU-S have a finite self-replication capacity which under normal circumstances exceeds the life span of the mouse. Chemotherapy or irradiation can damage the replicative capacity of CFU-S with bone marrow failure as a late sequela.

Granulocyte-Macrophage Progenitors (CFU-GM)

Shortly after the development of the CFU-S assay, Bradley and Metcalf (*27*) as well as Pluznik and Sachs (*28*) demonstrated that bone marrow cells would, with appropriate stimuli, form discrete colonies in semisolid media *in vitro*. These colonies arose from individual cells, and the cells giving rise to these colonies were designated colony-forming units in culture or CFU-C. The proliferation of these cells requires the presence of a colony-stimulating activity (CSF), and in 7 days colonies containing both granulocytes and monocyte-macrophages are formed. A single cell can give rise to both granulocytes and macrophages (*29*), and this cell has subsequently been renamed CFU-GM for colony-forming unit, granulocyte-macrophage. The CFU-GM is a separate cell population from the CFU-S, has a relatively high proliferation rate and little capacity for self-renewal (*15*). Conditions that demand granulopoiesis result in increased serum and tissue CSF levels as well as increased marrow CFU-GM numbers while cytotoxic agents cause decreased CFU-GM numbers (*30*). The CFU-GM assay is available for most mammalian species including man and has led to the identification of factors having both positive and negative effects on granulopoiesis.

Erythroid Progenitors (CFU-E, BFU-E)

More recently, it has been reported that the addition of erythropoietin to bone marrow cultures will stimulate the growth of colonies that contain hemoglobulin (*31*). Further study has shown that these erythroid colonies contain at least two classes of stem cells, a more primitive erythroid burst-forming unit (BFU-E) requiring long culture periods (10-14 days) with high erythropoietin levels and a more differentiated colony-forming unit-erythroid (CFU-E) requiring lower erythropoietin level and only 2 days of culture (*23,33*). Marrow CFU-E numbers follow a predictive pattern, increased following bleeding (*34*) and decreased during plethora induced by red cell transfusions (*35*). Erythroid progenitors as measured by clonal assays are sensitive to cytostatic agents (*30,36,37*) and known marrow toxicicants such as benzene (*38,39*). Use of these assays has revealed that some patients with aplastic anemia possess a population of lymphocytes capable of suppressing *in vitro* erythroid-colony formation by normal human bone marrow (*40*), whereas in congenital hypoplastic (Diamond-Blackfan) anemia, lymphocytes are normal, and the defect appears to reside in the erythroid progenitors (*41*). Thus these assays allow not only study of erythroid progenitor cell numbers but can also contribute to our understanding of hormonal factors and cell to cell interaction as may occur in disease states. Another technique used to study erythropoiesis is ^{59}Fe uptake in spleen and marrow which provides a specific marker for hemoglobin synthesis (*15,33*). In this assay, mice are killed 18 hr after injection of the isotope and tissues collected for counting. Further studies are needed, however, to determine the correlation between ^{59}Fe uptake and the clonal assays for erythroid precursors.

Other Clonal Bone Marrow Assays (CFU-BL, CFU-TL, CFU-M)

Clonal marrow assays have been developed for B-lymphocytes (CFU-BL), T-lymphocytes (CFU-TL), and megakaryocytes (CFU-M) by adding appropriate stimuli to semi-solid marrow cultures (*15,38-40*). These new model systems should allow further understanding of granulopoeiesis and may represent sensitive indicators of toxicity but need to be further examined.

Effects of Environmental Agents on Myelotoxicity

Benzene is one of the more widely known myelotoxic agents where short term exposure will induce profound pancytopenia and anemia (*41-43*). Following benzene exposure, there is decreased ^{59}Fe uptake by hematopoietic organs (*41*), but whether this compound acts at the more primitive BFU-E or the more mature CFU-E has not been defined.

Mercury has been reported to cause pancytopenia in man. *In vitro* studies have shown that micromolar concentrations of both inorganic and organic mercury inhibited colony formation in primary culture of mouse bone marrow (*46*). Addition of a toxic chemical to marrow cultures may suggest myelotoxicity but further confirmation by *in vivo* studies are necessary.

We have used a mouse model to investigate immunotoxicity. Our general experimental design employs the female B6C3F1 hybrid mouse exposed to chemicals at sublethal concentrations and fractions thereof. A series of cell and humoral mediated immunoassays, in addition to clonal marrow assays are performed. This review deals principally with the latter by describing the effects of a variety of environmental pollutants on the pleuripotent hemopoietic stem cell (CFU-S) and the granulocyte-macrophage progenitor (CFU-GM).

Tetrachlorodibenzo-*p*-dioxin (TCDD) is an environmental pollutant that is ubiquitous, persists in the environment, and causes varying degrees of marrow hypocellularity in all species of animals studied to date (*47*). Mice were exposed to 1.0, 5.0 or 15 μg/kg body weight of TCDD pre- and postnatally by maternal dosing (*48*). As shown in Table 1, both 5 and 15 μg/kg dosage groups showed a significant reduction in marrow cellularity, CFU-S and CFU-GM. Hematology profiles and blood smears revealed a normocytic anemia in these mice (*48*); bone marrow toxicity was correlated with depressed immunologic and host resistance responses.

Polybrominated biphenyls (PBB) were accidently introduced in cattle and chicken feed in Michigan and resulted in widespread human exposure (*49*). Mice were exposed pre/postnatally by maternal dosing at 0, 1, 3 and 10 mg/kg maternal body weight (*49*). As shown in Table 1, there was no significant decrease in marrow cellularity or CFU-GM. The CFU-S in male mice at the highest dose was 80% of controls but the difference was not significant (*50*). At the 1 mg/kg level, the CFU-GM numbers were significantly enhanced. In general the study showed little or no marrow alterations and also only minimal alterations in immunological function tests.

Benzo(a)pyrene, a constituent of petroleum compounds and derivatives, has long been recognized as a potent mutagen and carcinogen. Mice were exposed to 50, 200 or 400 mg/kg benzo(a)pyrene by subcutaneous injection for 10 doses over a 14-day period. Bone marrow cellularity showed a significant increase but there was a significant and dose responsive reduction in both marrow CFU-S and CFU-GM (Table 1). These mice also showed a vari-

Table 1. The effect of environmental chemicals on bone marrow.

		Cell or colony numbers, % of controls		
Chemical	Dose, mg/kg	Marrow cellularity	Pleuripotent stem cells (CFU-S)	Granulocyte-macrophage progenitors (CFU-GM)
TCDD	0.001	100^a	ND	121
	0.005	83^b	68^b	90
	0.015	61^c	37^c	21^c
PBB	1	91	ND	125^b
	3	95	ND	111
	10	86	ND	108
B(a)P	50	115	80^c	85
	200	105	80^c	82^b
	400	120^b	51^c	59^c
DES	0.2	84	97	16^b
	2	74	88	65^c
	8	68	59^c	63^c
IND	1	112	108	118
	2	88	ND	122
	4	96	81	120

[a] Percent of controls, 7 mice/group.
[b] $P < 0.05$ vs. controls.
[c] $P < 0.01$ vs. controls.

ety of immunological defects, primarily of humoral immunity (*51*).

Diethylstilbestrol (DES) was formerly prescribed as a synthetic estrogen and also was used to fatten cattle. Human residues accumulated from ingestion of dairy and meat products have been found. Mice were exposed by subcutaneous injection to 0.2, 2 and mg/kg body weight for 5 consecutive days (*52*). There was a dramatic decrease in marrow cellularity, CFU-S and CFU-GM (Table 1). These animals also exhibited highly enhanced sensitivity to *Listeria monocytogenes* and other generalized immunotoxic responses (*53*).

Indomethacin (IND) is a potent anti-inflammatory agent that appears to act by inhibiting prostaglandin synthetase. Mice were exposed to 0, 1, 2 and 4 mg/kg by subcutaneous injection for six consecutive days. In general there tended to be some enhancement of CFU-GM with no significant alterations in marrow cellularity or CFU-S (Table 1). These animals showed enhanced resistance to *Listeria monocytogenes* (*54*).

Mice exposed to orthophenylphenol or tris(2,3-dichloropropyl) phosphate exhibited no alteration in bone marrow cellularity or CFU-GM numbers (*55*).

Conclusions

Clonal marrow assays have greatly aided our understanding of hematopoiesis and factors controlling the production of blood cells. These assays are currently finding widespread use in cancer chemotherapy centers as tools to understand and to monitor myelotoxicity following antineoplastic therapy. We have shown that some environmental pollutants likewise have effects on marrow cellularity and progenitor cells and that these effects are readily detected by clonal marrow assays. It would appear that wider application of these techniques can help define myelotoxicity resulting from chemical exposure.

Anemia is commonly reported in long term chronic toxicity tests. This would suggest that myelotoxicity assessment panels should also include assays for erythroid progenitors. Erythropoiesis can also be assessed by *in vitro* clonal assays or by ^{59}Fe uptake *in vivo*.

Recently it has been shown that hemopoietic stem cells have a limited renewal capacity and that damage to these cells can induce a permanent decrease in their proliferative capacity (*20*). This residual marrow damage would not be predicted by parameters such as bone marrow cellularity, peripheral blood counts or even in some cases by CFU-S numbers (*20,36*). However "stressors" such as serial transfer of marrow (*20*), anemia (*21*) or irradiation (*19*) have revealed that these mice have a more limited hemopoietic response than controls. With the plethora of environmental chemicals it seems possible that exposure to one chemical may induce residual marrow damage that would make an individual more susceptible to a second chemical insult, although this hypothesis remains to be tested.

The authors greatly appreciate the assistance of Ms. Beth deBrito in the preparation of the manuscript.

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Contributed Article

Environmental Health Perspectives
Vol. 43, pp. 139-168, 1982

Report of the Federal Panel on Formaldehyde*

The Federal Panel on Formaldehyde concluded that definitive experiments exist which demonstrate the mutagenicity and carcinogenicity of formaldehyde under laboratory conditions. Formaldehyde induces both gene mutations and chromosomal aberrations in a variety of test systems. Inhalation of formaldehyde causes cancer of the nose in rats. The concentrations of formaldehyde in inhaled air that caused nasal cancer in Fisher 344 rats are within the same order of magnitude as those to which humans may be exposed. The data presently available do not permit a direct assessment of the carcinogenicity of formaldehyde to man. Epidemiologic studies on exposed human populations are in progress and may further clarify the situation. Other experimental and human studies on toxic effects such as teratogenicity and reproductive disorders are as yet inadequate for a health risk assessment.

The CIIT 24 month study on animal carcinogenicity has not yet been completely evaluated. Additional data are expected on the effects of prolonged exposure to lower doses of formaldehyde and on the possible carcinogenicity of formaldehyde in the mouse. The panel recommends that, for a comprehensive health risk assessment, further experiments be conducted on the effects of other modes of exposure (ingestion and skin penetration), the effects in humans, and on the pharmacokinetics of formaldehyde in man and animals and the possible role for formaldehyde in reproductive and chronic respiratory disorders.

It is the conclusion of the panel that formaldehyde should be presumed to pose a carcinogenic risk to humans.

Introduction

The first evidence that formaldehyde might represent a carcinogenic risk for man was obtained in October 1979 from an ongoing animal experiment conducted by Battelle Columbus Laboratories for the Chemical Industry Institute of Toxicology (CIIT) (*1*). The preliminary results were reviewed by Federal Government scientists, who concurred with the CIIT conclusions. As the experiment progressed and more data became available, the need for a full review of the potential health risks to humans from chronic exposure to formaldehyde became evident.

To accomplish this review, a panel of scientists from within the Federal Government was formed in April, 1980, under the auspices of the National Toxicology Program and coordinated by the Consumer Product Safety Commission. The panel members reviewed and evaluated the available published and unpublished information on the adverse health effects from repeated exposure to formaldehyde. Acute toxic effects and hypersensitivity were not considered, since they had recently been assessed by the Committee on Toxicology of the National Academy of Sciences (*2*).

Nearly seven billion pounds of formaldehyde are produced each year in the United States (*3*), most in the manufacture of urea-, phenol-, acetal-, and melamine-formaldehyde resins. The remainder is used to make other industrial chemicals, agriculture products, leather products, to preserve cosmetics, and to prepare vaccines, drugs, fumigants, and disinfectants. The formaldehyde-based resins are used as adhesives in the manufacture of particle board, veneers, and plywood, and in the production of insulating materials, plastics, protective coatings, textiles, paper, and rubber products.

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*Panel members: Richard A. Griesemer (Chairman), National Cancer Institute (now at Oak Ridge National Laboratory); Andrew G. U amer, Consumer Product Safety Commission; Joseph C. Arco Environmental Protection Agency; James R. Beall, Department of Energy; Aaron E. Blair, National Cancer Institute; Thor s F. X. Collins, Food and Drug Administration; Frederick J de Serres, National Institute of Environmental Health Sciences; Richard B. Everson, National Institute of Environmental Health Sciences; John F. Gamble, National Institute for Occupational Safety and Health; David W. Gaylor, National Center for Toxicological Research; David H. Groth, National Institute for Occupational Safety and Health; Han K. Kang, Occupational Safety and Health Administration; Richard A. Keer side, National Institute for Occupational Safety and Health; J. W liam Lloyd, Occupational Safety and Health Administration; Paul Nettesheim, National Institute of Environmental Health Sciences; Umbe o Saffiotti, National Cancer Institute; Eliz eth K. Weisburger, National Cancer Institute.

Under certain conditions, formaldehyde may be released from some of these products, creating the potential for human exposure to the chemical. Its presence in building materials, such as insulation, particle board, and plywood, as well as in other products, has resulted in complaints to the federal regulatory agencies. For example, the Consumer Product Safety Commission (CPSC) has received a number of complaints from consumers concerning adverse health effects associated with the release of formaldehyde gas from urea-formaldehyde foam insulation (*4*). Consumers have also expressed concern that the high concentrations of formaldehyde within their mobile homes may be unhealthy (*5*). Complaints such as these arise because formaldehyde causes irritation of the eyes, nose, throat, and skin, persistent cough, shortness of breath, nausea, headaches, and dizziness. Similar symptoms have been reported by workers who were exposed to formaldehyde resins that are used in the wood, textile, rubber and leather industries.

In October 1979, federal regulatory and research agencies became greatly concerned about the exposure of humans to formaldehyde when representatives of the Formaldehyde Institute reported preliminary findings from a carcinogenicity study showing that rats exposed to 15 ppm of formaldehyde developed squamous cell carcinoma of the nose (*1*). This concern was reinforced in January, 1980, when representatives of the Interagency Regulatory Liaison Group (IRLG) reviewed the carcinogenicity data in detail (*6*). As a result, CPSC, in cooperation with the IRLG agencies, convened a panel of scientists from eight federal agencies under the auspices of the National Toxicology Program to review health data related to formaldehyde. As part of this review, the panel members were asked to consider the following questions (*7*):

- Is there evidence indicating that formaldehyde may be tumorigenic/carcinogenic at doses other than 15 ppm in the CIIT study?
- Are there confounding factors in the CIIT study such as the irritating properties of formaldehyde, viral infection, special susceptibility of the rat to irritants, or protocol defects? If so, what are the relative merits of these factors?
- What conclusions can be drawn from the tumorigenic/carcinogenic results in the CIIT study?
- What is the applicability of the conclusions drawn in the CIIT study to the human situation?
- What is the relation of animal data from other irritant carcinogens to the human situation?
- How do other formaldehyde studies in animals and epidemiological studies affect conclusions about the human carcinogenicity of formaldehyde?
- Do short-term mutagenicity data support findings of carcinogenicity?
- What conclusions can be reached concerning the potential human carcinogenicity of formaldehyde?
- Are there additional data needed?
- Are these findings relevant to exposure from other routes?
- Is there evidence that formaldehyde is teratogenic or causes reproductive effects?

To address these questions the panel evaluated the published literature and available data from ongoing studies. This report is the result of the panel's review.

Chemistry and Metabolism

Chemistry

Formaldehyde, a one-carbon compound with the formula HCHO, has a molecular weight of 30.3 and a boiling point of 19°C. In the gaseous state formaldehyde is a highly reactive chemical with a characteristic pungent odor. It is readily soluble in nonpolar solvents such as chloroform, ether, or toluene but undergoes solvation in polar solvents such as water or methanol. Synthesis of formaldehyde is accomplished typically by the oxidation of methanol in the presence of a copper or silver catalyst

The common commercial form of formaldehyde (formalin) contains 37–50% formaldehyde in water by weight and is stabilized against polymerization by the addition of 1–15% methanol. Formaldehyde is also commercially available as a solid linear polymer containing 91–95% formaldehyde and 5–9% water (paraformaldehyde or polyoxymethylene) or as a cyclic polymer (trioxane or trioxymethylene). Trioxane, unlike polyoxymethylene, does not readily undergo depolymerization under most conditions.

Formaldehyde undergoes the following chemical reactions in biological systems (*8*):

Hydration in the presence of water:

$$HCHO + H_2O \longrightarrow CH_2(OH)_2 \qquad (1)$$

Reaction with the active hydrogen of ammonia, amines, or amides:

$$RNH_2 + HCHO \rightarrow RNHCH_2OH \qquad (2a)$$

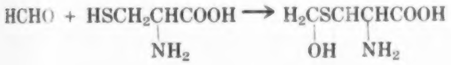
$$RNHCH_2OH + RNH_2 \rightarrow RNHCH_2NHR + H_2O \qquad (2b)$$

$$RCONH_2 + HCHO \rightarrow RCONHCH_2OH \qquad (3a)$$

$$RCONHCH_2OH + RCONH_2 \rightarrow RCONHCH_2NHOCR + H_2O \qquad (3b)$$

These reactions are of particular concern because of the ubiquity of nitrogen compounds (DNA, RNA, proteins, amino acids, etc.) in all biological systems. The reaction with purines and other amines produces an intermediate methylol product which is labile. The reaction product with a second amine moiety is stable.

Reaction with other compounds having active hydrogens such as thiols, nitroalkanes, hydrogen cyanide, and phenol:



$$HCHO + HSCH_2\underset{NH_2}{CH}COOH \rightarrow H_2\underset{OH}{C}SCH_2\underset{NH_2}{CH}COOH \qquad (4)$$

Condensation with HCl (and possibly other inorganic chlorides) in the presence of water to form bis(chloromethyl) ether:

$$2HCHO + 2HCl \rightarrow ClCH_2OCH_2Cl + H_2O \qquad (5)$$

Metabolism

Formaldehyde can enter the body through inhalation, ingestion or dermal absorption. Absorption of formaldehyde through the upper respiratory tract in dogs has been estimated to exceed 95% of the inhaled dose (*9*). Following oral exposure of dogs to formaldehyde, formate levels in blood increased rapidly indicating rapid uptake and metabolism (*10*). Dermal absorption has also been demonstrated (*11*) but does not appear to be significant in comparison to inhalation or ingestion.

Formaldehyde that enters the body appears to be converted rapidly to formate (*10, 12*) or to combine with tissue constituents by the reactions described above. The conversion of formaldehyde to formate occurs following intravenous (IV) infusion, subcutaneous injection, gastric intubation, or inhalation. Studies using IV infusion of 0.2*M* formaldehyde into dogs have shown that only a small amount of formaldehyde appears in the plasma during exposure (*10*). This becomes undetectable within 1 hr after cessation of infusion. The peak formate concentration following formaldehyde infusion was the same as when formate (0.2*M*) itself was infused. The plasma halflife for formate (between 80 and 90 min) was also similar. Formaldehyde could not be detected after oral administration of 0.2*M* formaldehyde in the same study while formate increased rapidly in the plasma with a halflife of 81.5 min.

Similar experiments on cynomolgus monkeys, in which 0.2*M* formaldehyde was infused IV, likewise showed no accumulation of formaldehyde in blood (*12*). The blood halflife was estimated to be 1.5 min. Similar halflives for blood formaldehyde have been observed in rats, guinea pigs, rabbits and cats (*13*). In a somewhat different experiment, McMartin et al. (*14*) administered ^{14}C-methanol by gastric intubation. Again, formaldehyde could not be detected although formate levels increased rapidly. A study in which humans were exposed to formaldehyde gas (0.78 mg/m^3) for 3 hr also demonstrated a rapid rise in blood and urine formate levels (*15*).

The rapid conversion of formaldehyde to formate occurred in many tissues in the various species examined, including human erythrocytes (*10*), liver and brain; sheep liver; rat brain, kidney and muscle; rabbit brain; and bovine brain and adrenals (*16*). The enzymes involved have been studied by Strittmatter and Ball (*17*) as well as by Uotila and Koivusalo (*16*). The oxidation is initiated by formation of *S*-formyl glutathione which is then oxidized by NAD and finally cleaved by a thiol esterase, releasing formic acid and glutathione. Formaldehyde has also been reported to be oxidized to formic acid by a nonspecific aldehyde dehydrogenase and by the tetrahydrofolic acid pathway (*18*).

Additional studies (*19*) have shown that following subcutaneous administration of ^{14}C-formaldehyde to rats, approximately 81% of the radioactivity appeared as CO_2. A small amount of the radioactivity was found in choline. Almost 60% of a subcutaneous dose of ^{14}C-formate similarly appeared as ^{14}C-CO_2, with small amounts of radioactivity in choline. Neely (*20*) administered formaldehyde intraperitoneally (IP) to rats and found that 82% of radiolabel was recovered as CO_2 and 13-14% as urinary methionine, serine and a cysteine adduct. At lower doses, only methionine was labeled. The author postulated that CO_2 was derived from serine (formed from glycine and N^5, N^{10}-methylene tetrahydrofolate) by deamination to pyruvate and oxidation in the Krebs cycle. The formation of methionine from ^{14}C-formaldehyde and homocysteine had previously been demonstrated by Berg (*21*). Formation of methionine would also account for the labeled choline observed by DuVigneaud et al. (*19*)

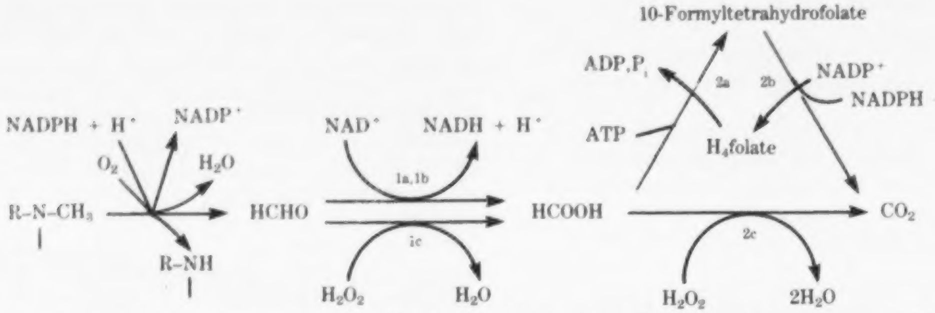

10-Formyltetrahydrofolate
ADP,P$_i$
ATP
2a
2b
NADP$^+$
NADPH + H$^+$
H$_4$folate
NADPH + H$^+$
NADP$^+$
O$_2$
H$_2$O
R-N-CH$_3$
R-NH
HCHO
NAD$^+$
NADH + H$^+$
1a,1b
1c
H$_2$O$_2$
H$_2$O
HCOOH
2c
CO$_2$
H$_2$O$_2$
2H$_2$O

FIGURE 1. Simplified reaction sequence from drug N-demethylation (cytochrome-P-450-dependent monooxygenase) to formaldehyde, formate, and CO_2 production. Reactions are: 1a, formaldehyde dehydrogenase (GSH); 1b, aldehyde dehydrogenase; 1c, catalase (peroxidatic mode); 2a, 10-formyltetrahydrofolate synthetase; 2b, 10-formyltetrahydrofolate dehydrogenase; 2c, catalase (peroxidatic mode).

via methylation of phosphatidyl ethanolamine. More recent work by Pruett et al. (*22*) has demonstrated the incorporation of ^{14}C-formaldehyde into the nucleic acid and protein fractions of WI 38 human diploid fibroblasts. Most of the radiolabel was found in RNA with lesser amounts in DNA and protein. The purine bases of both DNA and RNA were most heavily labeled.

In addition to the serine pathway to CO_2 postulated above (*20*), two other pathways have been identified. These are shown in Figure 1 from Waydhas et al. (*23*).

Waydhas et al. (*23*), McMartin et al. (*14*) and Palese and Tephly (*24*) have demonstrated that the catalase reaction (Fig. 1) is not of major importance and that the primary pathway to CO_2 from formate occurs via the tetrahydrofolic acid pathway. This has been demonstrated in rat liver perfusates (*23*), monkeys (*14*) and rats (*24*). Since the tetrahydrofolic acid pathway (Fig. 2), from Kitchens et al. (*2*), can

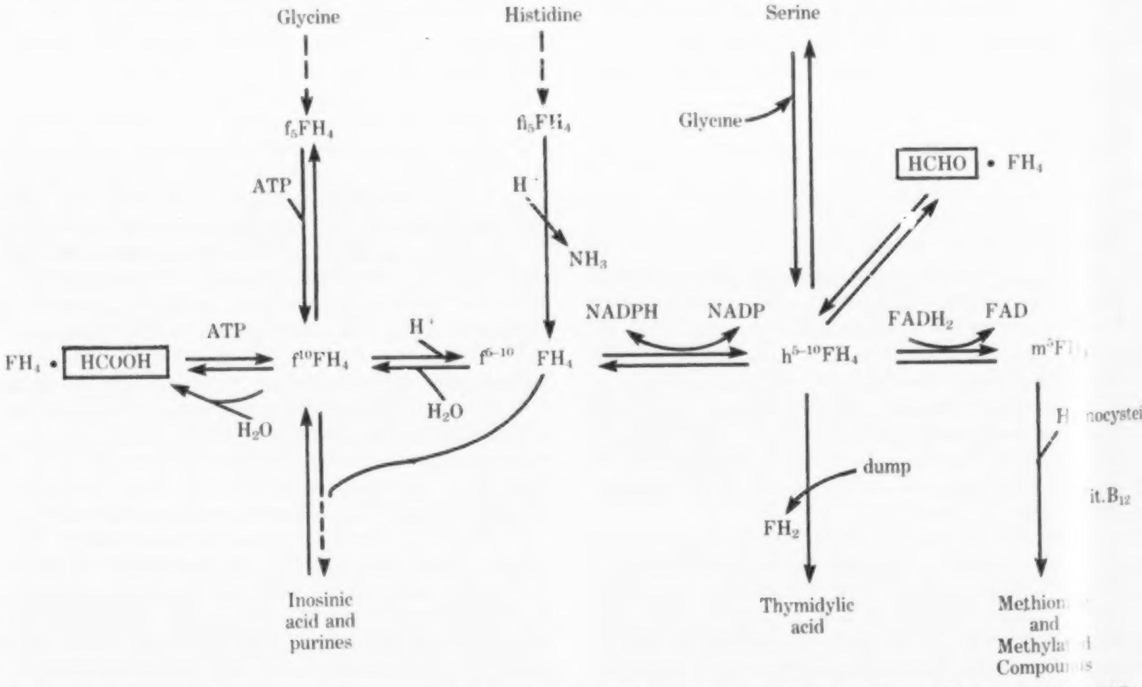

Glycine
Histidine
Serine
f$_5$FH$_4$
fi$_5$FH$_4$
Glycine
HCHO • FH$_4$
ATP
H$^+$
NH$_3$
ATP
H$^+$
NADPH
NADP
FADH$_2$
FAD
FH$_4$ • HCOOH
f^{10}FH$_4$
f$^{5-10}$
FH$_4$
h$^{5-10}$FH$_4$
m^{5}F
H$_2$O
H$_2$O
dump
FH$_2$
H
ocystei
it.B$_{12}$
Inosinic acid and purines
Thymidylic acid
Methion
and
Methyla
Compou

FIGURE 2. Sources of formate. FH_4 = tetrahydrofolic acid; $f^{10}FH_4$ = N^{10} formyltetrahydrofolic acid; f^5FH_4 = N^5 formyltetrahydrofolic acid; $f^{5-10}FH_4$ = $N^5 - N^{10}$ methylnyltetrahydrofolic acid; fi^5FH_4 = N^5 formininotetrahydrofolic acid; $h^{5-10}FH_4$ = N^{10} methylenetetrahydrofolic acid.

lead to the transfer of the carbon from formate to a number of other compounds (including serine), it is not clear that the 10-formyltetrahydrofolate dehydrogenase reaction (Fig. 1) is the only reaction of importance for CO_2 production in this pathway.

The sources of formate in Figure 2 include the degradation of amino acids such as histidine and tryptophan, and possibly serine and glycine as well. Formaldehyde or formate can also result from *N*-demethylation of drugs such as aminopyrine or ethylmorphine (*23*, *25*) or the metabolism of dihalomethanes (*26*).

Formaldehyde can be formed in mammalian tissues (pig brain and rat kidney) by enzymatic hydrolysis of 5-methyl tetrahydrofolate, in a reaction that appears to require the presence of biogenic amines. The reaction between the amine and formaldehyde is nonenzymatic and leads to the formation of various alkaloids (*27*). A similar reaction involving N^5, N^{10}-methylene tetrahydrofolate is found in human lymphocytes, brain, kidney, and platelets (*28*); it also apparently requires an amine acceptor. Formaldehyde can additionally result from the action of mixed function oxidases on the *N*-methyl groups of various xenobiotics (*29*). The authors report, however, that the rate of oxidation of formaldehyde to formate is higher than the rate of formation of formaldehyde in the above reaction.

Finally, whereas the conversion of formaldehyde to CO_2 occurs in a similar manner in the different species studied, the relative importance of each reaction differs among species and tissues. Thus, the rat is able to convert formate to CO_2 at more than twice the rate of monkeys (or man) and, as a result, has lower blood formate levels (*14*) and does not excrete formate in the urine (*20*). Man additionally possesses 50% more hepatic dehydrogenase than do rats (*30*). With regard to tissue differences, Den Engelse et al. (*31*) have shown that mouse (C3Hf/A) and hamster (Syrian Golden) lungs do not convert formate to CO_2 as efficiently as liver tissue does.

In summary, free formaldehyde is not usually found in plasma or other body tissues in measurable quantities, such endogenous formaldehyde as is produced may be reasonably presumed to be metabolized rapidly to formate or to enter the one carbon pool. When exogenous exposure does occur, formaldehyde is likewise rapidly metabolized to formate and excreted, converted to CO_2 and/or incorporated into other molecules. The same paths seem to occur in all mammalian species examined to date, but reaction rates differ among various species and tissues. Neither the ratio of metabolic deactivation, to tissue or small molecule binding, nor the effect of route of exposure on this ratio is known at this time. Thus, although Egle's work (*9*) suggests that the respiratory tract is the primary area at risk for tumor development, other body sites cannot be ruled out.

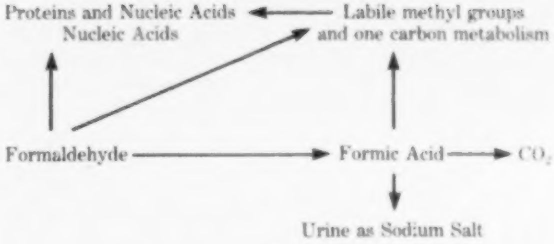


FIGURE 3. Overall metabolism of formaldehyde.

The overall metabolism of formaldehyde is summarized in Figure 3 (adapted from Kitchens et al.) (*8*).

As can be seen from Figure 3, formaldehyde is more chemically active than any of its direct metabolites and would therefore appear to be the chemical substance of most concern for carcinogenicity. The possibility exists, however, as proposed by Poverenny et al. (*32*), that the actual carcinogenic agent may be an amino acid (or other) adduct.

Teratology and Reproduction

Reproduction and Teratology Studies in Animals

The effects of formaldehyde or hexamethylenetetramine (HMT) on teratology and reproduction in laboratory animals have been studied using four methods of administration: inhalation, oral gavage, dietary, or in drinking water. Because the method of administration might have affected the results, the studies are presented chronologically for each method. Hexamethylenetetramine (HMT), an antimicrobial food additive, degrades to formaldehyde and ammonia in an acid medium or in the presence of protein, as in the digestive tract (*33*).

Inhalation Studies. Gofmekler and his colleagues studied the teratogenic and toxic effects of formaldehyde in 36 female rats (12 per group) exposed to 0, 0.012 or 1.0 mg/m^3 from 10 to 14 days before impregnation through gestation. Three male rats per dose level were also exposed for 6-10 days before they were mated.

Gofmekler (*34*) reported the effect of formaldehyde on fertility, fetal weights and organ weights. In both treated groups, formaldehyde caused a 14-15% increase in the length of gestation; it also increased the average body weight of the offspring and their heart, adrenal, and kidney weights. These changes might have resulted from the extended gestation time. In contrast, the liver and lungs from pups in the treated groups weighed less than those of the control pups. Although Gofmekler stated that there was a decrease in the litter size, his data suggest that this may not be accurate. Our calculations, based on data in Gofmekler's article, show that groups exposed to 0.012 and 1.0 mg/m^3 of formaldehyde had average litter sizes of 19.6 and 17.3 pups, respectively, as compared to the control value of 11.2. These calculations assume that all females in each group became pregnant. Gofmekler stated that some females in each group did not become pregnant, but did not report the details. This implies that several dams produced over 20 pups each.

Gofmekler, Pushkina and Klevtsova (*35*) reported data related to changes in the developing embryo. Identical data were reported by Pushkina, Gofmekler, and Klevtsova (*36*). They measured ascorbic acid in whole embryo, placenta, maternal liver and fetal liver; "nucleic acids" (apparently RNA) in maternal liver, fetal brain and fetal liver; and DNA in maternal and fetal liver. Significant decreases in ascorbic acid concentrations occurred in the whole embryo and in the maternal liver at both dose levels. A significant increase in ascorbic acid concentration in liver occurred in offspring from dams exposed to 0.012 mg/m^3. RNA concentrations in maternal livers were greater at both dose levels than they were in controls. RNA concentrations of fetal brain were similar in control and treated groups. DNA content was significantly lower in maternal and fetal liver in both treated groups than in control animals. The authors concluded that formaldehyde "significantly inhibited the synthesis of nucleic acids." This conclusion is not, however, supported by the data in the article.

Gofmekler and Bonashevskaya (*37*) reported the data related to histopathological changes in the liver and kidneys of fetuses from dams exposed to formaldehyde. The hepatic changes included an increased proliferation of epithelial cells in the bile duct and segmented forms in the hepatic sinusoids. The kidney changes included renal epithelial cells with polymorphic nuclei, casts in the lumina of some tubules, and functional alterations in the renal tubule apparatus. Exposure to 1.0 mg/m^3 of formaldehyde also caused a decrease in myocardial glycogen, an involution of the thymic lymphoid tissue and disintegration of lymphocytes. Histologically, the testes of adult males exposed to formaldehyde were similar to those of the controls.

From macroscopic evaluation it was reported that formaldehyde inhalation by pregnant dams did not affect embryonic or fetal development.

Sheveleva (*38*) studied the teratogenic potential of formaldehyde in pregnant albino rats. The rats were exposed to 0.005, 0.0005 or 0 mg/l of formaldehyde for 4 hr each day on days 1-19 of gestation. Fifteen females per group were killed on day 20, while six were kept to obtain progeny. On day 17 of pregnancy, body weight, spontaneous mobility, rectal temperature and hemoglobin, leukocyte and erythrocyte concentrations were measured. On day 20, corpora lutea, implantation sites, pre- and postimplantation deaths, and living fetuses were counted. The sizes and weights of living fetuses were determined.

Dams exposed to 0.005 mg/l of formaldehyde exhibited decreased neuromuscular excitability, spontaneous mobility, rectal temperature, and hemoglobin concentration. Exposure to 0.0005 mg/l only caused a slight increase in lymphocytes.

At sacrifice on day 20, the number of preimplantation deaths was higher in both groups exposed to formaldehyde than it was in the controls. Interestingly, the number of live fetuses was approximately the same in all groups. No external malformations were observed in the offspring.

Six dams from each group delivered offspring on day 22; all progeny appeared to be normal at birth. At one month postpartum, the female offspring from control dams were larger than the female offspring from treated dams. For male progeny, the opposite was true. At one month, the spontaneous mobility of progeny from treated dams was less than that of control progeny. By two months, the hemoglobin and leukocyte concentrations were decreased in progeny of dosed dams but not in a dose-related manner.

Guseva (*39*) studied the gonadotropic effects of formaldehyde by measuring testicular nucleic acid content in rats. During a 6 month treatment period, 3 groups of male rats were subjected to a combined action of formaldehyde orally (in drinking water) and by inhalation. Group 1 received formaldehyde at a concentration of 0.1 mg/l in drinking water and by inhalation at a level of 0.5 mg/m^3. Group 2 received formaldehyde at a concentration of 0.01 mg/l and by inhalation at a level of 0.25 mg/m^3. Group 3 received formaldehyde at a concentration of 0.005 mg/l and by inhalation at a level of 0.12 mg/m^3. Group 4 served as untreated controls. Treatment in the drinking water was continuous and simultaneous exposure to formaldehyde by both routes occurred five times per week for 4 hr each time. Reproductive function was evaluated by pairing each treated male with two virgin untreated females. Although the number of males was not specified, the use of six females per group implies the use of three males in each group. On day 20, an unspecified number of pregnant females were killed and their offspring were removed and examined. Gus va failed to mention the results of the examination of the fetuses. The remaining dams were allowed to produce offspring. The number and weight of newborn rats were recorded. Observations of their subsequent development extended over one month. The time of eye opening and other developmental indices were recorded for the offspring of males in groups 1 and 3 only.

The capacity of the treated males to produce offspring was not diminished. There was no effect on the weight of the fetuses or the size of the litters. The offspring were morphologically normal at birth and developed normally thereafter. Gonadotrophin levels were not significantly different between males in the control group and those in the treatment groups. However, the amount of nucleic acid in the testes of males of groups 1 and 2 was significantly less than the amount in the testes of the controls.

Sanotskii et al. (*40*) studied the effects of formaldehyde on reproduction in an unspecified strain and number of albino rats. They exposed groups of pregnant and nonpregnant rats to 0, 0.4 or 6.0 mg/m^3 of formaldehyde for 4 hr/day for 20 days. Evaluations were made of body weight, nervous system function, renal function, hepatic function, and biliary levels of cholic acid. Blood parameters, oxygen uptake, and rate of respiration were measured.

Nonpregnant animals were more susceptible to the effects of formaldehyde than were pregnant animals. Formaldehyde at 6.0 mg/m^3 resulted in altered renal function as shown by decreases in daily diuresis and urinary chlorides and an increase in urinary protein concentration. The increase in urinary protein may have reflected decreased urinary output. Altered hepatic function was manifested by a decrease in urinary excretion of hippuric acid. At 6.0 mg/m^3, only blood hemoglobin decreased in the pregnant rats. Exposure to 0.4 mg/m^3 did not produce toxic changes in either pregnant or nonpregnant rats.

All the studies suffered from an unreported or inadequate number of animals treated per dose level. In the teratology studies, detailed results from skeletal and soft-tissue analyses were missing. None of the studies had a sufficient number of dose levels to fulfill current requirements. Even with these deficiencies, there were indications that formaldehyde might affect reproductive potential by altering testicular nucleic acids and by increasing pre-implantation losses.

Dietary Studies. Hurni and Ohder (*41*) studied the effects of formaldehyde on reproduction in the beagle. Groups of 9-11 pregnant bitches were fed dietary levels of 600 or 1250 ppm HMT, or of 125 or 375 ppm formaldehyde, from 4 days after mating to day 56. Eleven control dogs ate unadulterated chow. The bitches were weighed weekly. The pups were weighed at birth and twice weekly thereafter. They were inspected for visible defects immediately after birth and at 8 weeks postpartum. Stillborn pups and those that died before weaning were autopsied and examined for internal and skeletal anomalies.

Neither formaldehyde nor HMT affected the pregnancy rate. Maternal body weights increased normally during pregnancy in all groups. The duration of gestation was not affected by formaldehyde or HMT. Mean litter sizes were within the normal range for all groups. In the group that received 1250 ppm of HMT, there was a greater percentage of stillborn pups than in any other group; this was mainly due to one litter in which 7 of 9 pups were dead. The stillborn pups were not malformed.

During the first month, the pups from bitches given 1250 ppm HMT grew less than normal. The retarded growth coincided with increased mortality. In the same group, the percentage of pups that survived to weaning was lower than it was in the other groups. All the dogs that were observed for up to 9 months exhibited normal behavior, appearance, mobility, and muscular coordination.

Natvig, Andersen and Rasmussen (*42*) studied the effects of HMT fed to Wistar rats. Two groups of 16 male and 16 female rats each were fed a diet containing 0.0 or 0.16% HMT starting at 2 months of age and continuing for three months, when they were mated with group mates. Their offspring were fed the same diet. The offspring were weighed at 7 and 15 weeks, measured for voluntary muscle activity at 6 weeks, and killed when they were 123 days old. Half of them were autopsied.

There were no detectable differences between the test and control groups. The fertility of the treated animals was similar to that of control animals. The offspring from both groups had similar muscular activity, body weights, general health and organ weights.

For the purpose of clarifying the teratogenic potential of formaldehyde, it is unfortunate that in both of these studies the dams were allowed to deliver naturally. Removing all the pups by cesarean section would have permitted detailed examination for anomalies. By allowing the dams to litter, it was possible that the malformed pups were eaten by the mother before being seen by the investigator. Neither report states if an observer was present at all deliveries. Although teratogenic effects were not noted, HMT appeared to have fetotoxic and postnatal effects in the beagle at 1250 ppm (31 mg/kg). In the rat study, additional dose levels would have been desirable.

Intubation Studies. Marks, Worthy and Staples (*43*) intubated pregnant outbred albino mice on days 6-15 of gestation with 1% aqueous formaldehyde at levels of 0, 74, 148, or 185 mg/kg/day. On day 18, the mice were killed and the offspring examined. All mice were coded in a manner that prevented laboratory personnel from knowing which ones belonged to the control and test groups. Visceral observations were made by the Staples technique and all fetuses were examined for skeletal anomalies.

Formaldehyde was toxic to 22 of 34 pregnant mice at 185 mg/kg. At 74 mg/kg, there was a significant decrease in average weight gain during pregnancy. Formaldehyde treatment, at any dose level, did not result in malformations.

Although formaldehyde caused toxicity in pregnant mice, it was not teratogenic. Because of the methods employed and the number of animals used at each level, this appears to be a valid, reliable, and negative teratogenic study of orally administered formaldehyde.

Drinking Water Studies. Della Porta, Cabral and Parmiani (*44*) conducted several studies on the effects of HMT in rats and published results in a single manuscript.

In the short-term reproduction and toxicity experiment, 12 females and 6 males were given a solution of 1% HMT in the drinking water, beginning at 8 weeks of age. Two weeks later, the animals were mated (2 females: 1 male) and treatment of the females was continued during pregnancy and nursing. Twenty-four males and 24 females were randomly selected from the progeny and treated as above until 20 weeks post partum. Each animal was weighed weekly. At the end of treatment, the animals were sacrificed, their organs were weighed, and histological examinations were conducted. Groups of 12 untreated dams and 48 pups were used as controls.

All treated females and 11 of 12 control females became pregnant and produced 124 and 118 offspring, respectively. The progeny of the treated females were not malformed. The mean body weights of the treated males and females were significantly less than those of controls. These differences lasted up to 9 weeks for males and to 13 weeks for females. After these times, body weights became comparable to the control values. When the offspring were autopsied at 22 weeks, no microscopic or macroscopic lesions were seen. Body weights and organ weights (liver, kidneys, spleen, thymus, pituitary, adrenals and testes) were similar for treated and control groups.

In the second experiment, two females and one male were given a solution of 1% HMT in the drinking water before mating. The treatment of females continued until two litters of 10 rats each had been weaned. Treatment of the F_1 progeny continued until they were 40 weeks of age. To produce an F_2 generation, seven F_1 females were mated with three F_1 males at 26 weeks of age. F_2 animals were mated at 15 weeks of age to produce an F_3 generation. F_1 and F_2 animals were given HMT until the 40th week postpartum; F_3 animals were given HMT to the 20th week. F_1, F_2 and F_3 animals were observed for 130 weeks (2.5 years). All survivors were sacrificed when they reached 3 years of age.

The survival rates of all the generations of offspring were not affected by treatment. Mean body weights, obtained many times during the experiments, showed no significant differences between control and treated groups.

In another study, five adult female rats were bred and treated with 2% HMT in the drinking water until 49 offspring were weaned. The offspring were treated with 2% HMT until they were 50 weeks of age. After treatment, the survivors were observed until 130 weeks of age. Rats (48 males and 48 females) from a previous study served as controls; they were observed until they were 3 years of age. Mean body weights were not significantly different between treated and control animals.

All three studies lacked a sufficient number of animals per dose level and number of dose levels to permit a meaningful evaluation of the potential of HMT to affect reproduction of fetal development. The third study also lacked appropriate concurrent control animals.

Reproduction and Teratology Studies in Humans

Shumilina (*45*) studied the reproductive potential of women who were exposed to formaldehyde. In the study, the menstrual and reproductive functions of 446 women were evaluated in two experimental groups. The first group of 130 women were fabric trim shop finishers. The second group of 316 women were fabric warehouse inspectors. A third group of 200 industrial goods saleswomen who were not exposed to formaldehyde were used as controls. The trim shop finishers worked in atmospheric concentrations of formaldehyde ranging from 1.5 to 4.5 mg/m^3. The warehouse inspectors worked where formaldehyde concentrations varied from 0.3 to 0.7 mg/m^3 in one shop and from 0.05 to 0.1 mg/m^3 in another shop. All women were given complete medical examinations.

Menstrual disorders were found in 47.5% of the women exposed to formaldehyde as compared to 18.6% of the controls. Dysmenorrhea was found in 24.3% of the finishers as compared to 9.2% of the controls and 20.2% of the inspectors. It occurred more frequently in the 31-40 year-old women (20.7% in the experimental group versus 6.7% in the controls). A cause of the painful menstruation was not found. Hypomenstrual syndrome occurred in 1.2% of the finishers with less than 5 years of potential exposure to formaldehyde, in 47% with 6-10 years of potential exposure, and in 6.8% of cases with more than 10 years of potential exposure. More menstrual disorders were found in workers below 30 years of age (33.4%), but fewer disorders were observed in persons aged 30-40 years (20.8%).

The incidence of inflammatory diseases in the reproductive tract among trim shop finishers was 38.2% and among the warehouse inspectors it was 25.1%. Both of these values were greater than the control value ($p < 0.05$). The percentage of women with exposure to formaldehyde who had primary sterility was 5.2; this value may be compared to 2.5% incidence in the controls. The incidence of secondary sterility was 15.3% in the finishers, as compared to 6.5% in the controls. The high incidence of secondary sterility in exposed women may have been due to previous inflammatory diseases.

The number of term deliveries and artificial abortions did not differ significantly among the groups of workers investigated. Anemia, the most frequent complication of pregnancy, occurred twice as often in women exposed to formaldehyde, as it did in control women. The hazard of intrauterine asphyxiation of the fetus was also encountered twice as often in the exposed women as in the control group. The percentage of children with low birth weights was greater in women exposed to formaldehyde than it was in the control women.

Menstrual disorders were found in women exposed to formaldehyde. Although the number of term births and artificial abortions did not differ among the groups, there were increases in primary and secondary sterility, anemia, and intrauterine asphyxiation of the fetus among women exposed to formaldehyde. Though these data have many limitations, they raise the possibility that formaldehyde may affect human reproductive processes. Additional research is needed to confirm these effects.

Reproduction-Related Studies

Palkovits and Mitro (*46*) studied formalin-induced stress in neonatal rats. They injected one group of newborn Wistar rats with 0.02 ml of 2% formaldehyde IP once on the day of birth. A second group was injected daily for the first 4 days after birth. Control animals were untreated. At 24 hr after the last injections, all neonates were decapitated. The adrenals and hypothalamus were examined microscopically.

In the neonates injected for 4 days, degenerative cellular atrophy occurred in the ventro-medial arcuate of the hypothalamus. Single injections of formaldehyde did not cause degenerative changes but did cause decreased cellular activity in the medial field of the ventromedial nucleus and the arcuate nucleus and an accumulation of granules in the neuronal cytoplasm. In both groups, formaldehyde injections increased nuclear volume in the adrenals. These changes indicate that the hypothalamus of the neonatal rat is sensitive to corticoid feedback induced by formaldehyde administration.

Cohen (*47*) studied the response to formaldehyde injection in fetal rats by measuring ascorbic acid levels in the adrenals. Pregnant female rats were anesthetized with ether and delivered by cesarean section. The first fetus from each litter served as the control Approximately 6 μl/g of 2% formaldehyde were then injected SC into one or more litter mates. Fetuses were injected with formaldehyde at 18.5, 19.5, 20.5 or 21.5 days of gestation.

Injections of formaldehyde resulted in decreased ascorbic acid levels in the adrenals at 20.5 days of gestation. Injections on other days of gestation did not cause this response in fetuses.

Conner et al. (*48*) studied the effects of formaldehyde injected into the uterus of pregnant rats. They were interested in its potential as a contragestational agent. On day 3 or day 7 after mating, they injected 0.05 ml of formaldehyde (40, 10, 7, 3.5, 2.0, 0.5, 0.05 or 0.0005%) into one uterine horn and 0.09% saline into the other horn (control) of 3-5 pregnant Sprague-Dawley rats/group. All solutions of formaldehyde also contained 12-15% methanol. On day 15, dams were sacrificed, and the number of corpora lutea, viable fetuses, and resorptions were counted.

Injections of 40 and 10% formaldehyde produced maternal toxicity and death. Injections of 7.0 through 0.5% on day 3 terminated most pregnancies. When these concentrations were injected on day 7, most pregnancies continued. The authors concluded that the contragestational properties of formaldehyde were similar to those of other protein denaturing agents, including ethanol, methanol and silver nitrate. Because methanol solutions alone were not tested, contragestational effects of methanol could not be clearly distinguished from those of formaldehyde.

These physiological and endocrine studies are interesting but their relation to the teratogenic potential of formaldehyde is unclear.

Dominant Lethal Studies

Epstein et al. (*49*) tested formaldehyde for mutagenicity by the use of the modified dominant lethal test. Male ICR/Ha Swiss mice were given a single dose of 16, 20, 32 or 40 mg/kg of formaldehyde IP. After dosing, each male was placed with three females weekly for 3 weeks. Alternatively, male ICR/Ha Swiss mice were given dose levels of 16 or 20 mg/kg IP and placed with three females weekly for 8 weeks. At the dose levels tested, formaldehyde did not produce an increase in early fetal deaths or pre-implantation losses.

Formaldehyde did not appear to affect spermatogenesis or fertility in mice at the dose levels tested in this study.

Recommendations

Teratology. The data are inadequate to demonstrate whether or not formaldehyde is teratogenic. The panel's survey revealed only one well-conducted study and that was done by oral administration in mice (*43*). Recommendations are that teratology studies should be performed in rats and rabbits and that these studies should include the administration of formaldehyde by inhalation.

Animal Reproduction. The study of Della Porta et al. of HMT (*44*) lacked the proper number of animals per group needed to evaluate reproductive and teratogenic effects. Multigeneration studies with a larger number of animals should be conducted to test the effects of formaldehyde on reproduction.

Although the physiological and endocrine studies contribute to an understanding of the reproductive effects of formaldehyde, many questions remain unanswered. Mechanisms of biochemical changes and teratology cannot be elucidated until additional biochemical and pharmacological studies are done including placental transfer studies.

Human Reproduction. Shumilina's study (*45*) revealed several possible effects of formaldehyde on human reproduction, but the study lacks sufficient data to answer the questions it raises. Controlled epidemiological studies should be conducted.

Mutagenicity

In a review of the extensive literature on the genetic toxicology of formaldehyde by Auerbach et al. (*50*), the results of experiments on formaldehyde-treated food in Drosophila are discussed as well as data obtained by exposure of other organisms to vapor or aqueous solutions of formaldehyde and by combination treatments with formaldehyde and other mutagens. Formaldehyde treatment alone gives positive but weak responses in many laboratory organisms; both gene mutations and various types of chromosome aberrations are produced. A brief summary of the genetic effects of formaldehyde has also been published by Cooper (*51*).

Genetic Effects of Exposure

Formaldehyde-Treated Food. The most extensive data (*50*) have resulted from treatment of Drosophila with formaldehyde-food (FF). In the early experiments of Rapoport (*52*) treatment of young larvae resulted in an increase of sex-linked recessive lethals from a control frequency of 0.2% to about 6%. FF produced all types of gene mutations (both point mutations and deletions) and chromosome rearrangements (small duplications and deficiencies to inversions and translocations). These results were independently confirmed by Kaplan (*53*). Slizynska (*54*) compared the structural changes induced by FF with those produced by x-rays. In contrast to 97% translocations or inversions and fewer than 3% small deficiencies or duplications induced by x-rays, only 30% translocations or inversions and 64% small duplications or deficiencies were found among FF-induced changes.

Formaldehyde Vapors. No mutations were produced by exposing Drosophila adults for up to 1 hr or larvae for up to 2 hr to sublethal concentrations of formaldehyde gas (*55*).

Formaldehyde Solutions. Early experiments in *Escherichia coli* B (*56*) showed that formaldehyde is a very weak agent for the production of point mutations from streptomycin-dependence to independence. Injection of formaldehyde solutions into adult Drosophila provided an effective means for inducing sex-linked recessive lethal mutations, but lower frequencies were found than obtained with FF (*57, 58*).

More recent studies have shown that the ability of formaldehyde to induce forward mutations at specific loci in the *ad*-3 region of Neurospora (by direct treatment at levels producing 75 - 5% survival) increased from a spontaneous frequency of 0.1 × 10^{-6} to 4 - 6 × 10^{-6} among survivors (*59*). With this assay, gene mutations resulting from both point mutations and multilocus deletions can be detected. Genetic characterization of formaldehyde-induced *ad*-3 mutants is in progress. Formaldehyde was found to induce point mutation by reverse-mutation in both Neurospora (*60, 61*) and Aspergillus (*62*), but not in Salmonella (*63, 64*). The National Toxicology Program has reported that, in recent investigations by three independent laboratories using the Ames test, formaldehyde tested positively in one of four bacterial strains (TA 100) that detects base pair substitution mutations.

A number of genetic effects have been found in mammalian cells in culture. These include: (1) an 8-10 fold increase in forward mutation frequency at the thymidine kinase (TK) locus in mouse lymphoma cells (*65*); (2) a 1.5–3-fold increase in the frequency of sister chromatid exchanges in Chinese hamster ovary cells and in human lymphocytes in culture (*66*) and (3) induction of unscheduled DNA synthesis in HeLa cells over a dose range of $10^{-6} - 10^{-8}$ *M* (*67*).

Mammalian* in vivo *Studies. No data were found in the literature on the induction of genetic damage in mammals either in somatic cells or germ cells. Epstein et al. (*68*) were unable to detect early fetal deaths induced by formaldehyde injection in an assay for dominant lethality in mice.

Interaction with Other Mutagens. In Drosophila, Sobels (*69*) found that either pre- or post-treatments with formaldehyde almost doubled the frequencies of x-ray-induced sex-linked recessive lethals. In Neurospora, early experiments by Dickey et al. (*70*), Kolmark (*71*), and Kolmark and Westergaard (*72*) showed that formaldehyde by itself is only weakly mutagenic, whereas a mixture of formaldehyde and peroxide gave a much higher effect than could be predicted on the basis of a strictly additive effect of the two agents.

Increased Effects in Repair-Deficient Strains. Nishioka (*73*) compared the lethality and mutagenic damage produced by formaldehyde in *E. coli* strains HCR+ and HCR- and found that, in the latter excision-repair deficient strain, much higher levels of effect were produced. Comparisons of the ability of direct treatment with formaldehyde solutions to induce specific locus mutations in the *ad*-3 region of wild-type and excision-repair deficient, two-component heterokaryons of Neurospora have shown up to a 370-fold higher mutation frequency in the excision-repair deficient strain (*59*). The same is true for excision-repair deficient mutants of *Saccharomyces cerevisiae* (*74*). In addition, more pronounced genetic effects of formaldehyde were found with cell lines of *Xeroderma pigmentosum* as compared with normal human fibroblasts in culture (*75*).

Summary

Formaldehyde produces gene mutations and such chromosome aberrations as deficiencies, duplications, inversions, and translocations; no data are available for the production of aneuploidy. In most experiments, dose-response relationships could only be demonstrated with difficulty or not at all, and all data are consistent with the classification of formaldehyde as a weak mutagen. Formaldehyde has been shown to interact with other mutagens such as x-rays, ultraviolet radiation and hydrogen peroxide, and to at least double the frequencies of mutants expected from treatment with these agents. Formaldehyde effects on both lethality and the induction of mutations in microbes are markedly enhanced in excision-repair deficient strains. No data are available from studies on mammalian systems *in vivo* on genetic effects on germ cells.

Risk Assessment

Since there are no effects reported on mammalian germ cells the genetic risk of exposure to formaldehyde can only be made by extrapolation from experiments with other laboratory organisms. The only genetic data relevant for risk estimation are those for the induction of gene mutations and chromosome aberrations. There are no data on the induction of aneuploidy by nondisjunction or any other mechanism. In this respect, the most extensive data result from experiments with Drosophila on formaldehyde-treated food. It is not known how representative these data are of genetic effects that would be expected from exposures to vapors or solutions. The Drosophila data show that 64% of the transmitted chromosomal effects studied resulted from small duplications or deficiencies, the remainder were due to large inversions and translocations. No data were found which would be useful in determining whether a comparable spectrum of genetic alterations would be found with lower chronic levels of exposure. In conclusion, although heritable genetic damage has been demonstrated, only small increases would be expected from the levels of formaldehyde which might reach the germ cells. The Drosophila data, for example, indicate that sensitivity is limited to effects on males and is restricted to early spermatocytes; whether similar effects can be expected in whole animal systems is unknown.

Recommendations for Future Studies

The genetic effects of formaldehyde in man could be more readily predicted if selected experiments were performed on laboratory animals. Such studies could begin with an experiment using the *in vivo* mammalian spot test to determine whether formaldehyde vapors or solutions would cause gene mutations in somatic cells that affect coat color (*76*). This test has the advantage of requiring exposure and analysis of progeny from only 30-50 pregnant females. The test is thus relatively inexpensive and has been used to evaluate the mutagenic activity of a wide range of chemical agents.

In the event of a positive result in the mammalian spot test, two additional assays should be applied in mice: (1) the heritable translocation test (*77*) and (2) the morphological specific locus test (*78*). Considerably more work is involved in these two assays but they are useful to determine, respectively, whether heritable chromosome aberrations and gene mutations have been produced. In the presence of a negative spot test it is unlikely that positive results would be found in the latter two tests which detect genetic effects produced in male germ cells.

Carcinogenicity

The literature search revealed only a few studies that were designed for the purpose of testing formaldehyde for carcinogenic activity. Because of limitations in experimental design, many of the reported studies are inconclusive. In this section only those studies will be reviewed which were conducted over a sufficient length of time to permit detection of carcinogenic activity activity of the test chemical. The investigations will be discussed in order of their importance as judged by this panel, starting with the inhalation exposure studies.

Review of Formaldehyde Carcinogenesis Studies

By far the most important carcinogenesis study conducted to date with formaldehyde is the CIIT inhalation study recently reported by Swenberg et al. (*79*). F344 rats and B6C3F$_1$ mice (120 male and 120 female rats or mice per group) were chronically exposed to 2.1, 5.6 and 14.1 ppm of formaldehyde vapors for 6 hr/day, 5 days/week. During the first 18 months of exposure, 2, 2, 8 and 44 rats died in the groups exposed to 0, 2.1, 5.6 and 14.1 ppm of formaldehyde, respectively. Of the 44 rats which died spontaneously in the group exposed to 14.1 ppm, 32 had nasal tumors (28 squamous carcinomas and four squamous papillomas). Many of the rats also showed hyperplastic and metaplastic lesions of the nasal epithelium. Hyperplastic and metaplastic lesions were also detected in rats exposed to 5.6 ppm formaldehyde (14 lesions were found in eight rats), indicating that the nasal epithelium was significantly damaged at this lower level of exposure. At 18 months of exposure, 40 rats were killed in each group. Eight of 40 rats exposed to 14.1 ppm had developed nasal squamous carcinomas. Although no carcinomas of the airways were found in the other groups, one adenomatous polyp was found in one rat of each of the groups exposed to 2.1, 5 and 14.1 ppm. In addition, a large number of hyperplastic and metaplastic lesions was detected in the nasal epithelium of these rats, even at formaldehyde concentrations as low as 2.1 ppm. No exposure-related tissue abnormalities were reported at sites other than the nasal cavities and no exposure-related neoplasias were reported in formaldehyde exposed mice, but examination of the animals sacrificed after 24 months of exposure is not complete.*

One complication noticed during the study was a spontaneous outbreak in rats of sialodacryoadenitis. The evidence for this consisted of decreased body weight in all dosed and control rat groups at about the 52nd week of the experiment, followed by prompt recovery of body weight, and histopathological demonstration of typical lesions in lacrimal and salivary glands and in the upper respiratory tissues of dosed and control rats in the 12 month sacrifice groups. Evidence of sialodacryoadenitis was not found in rats sacrificed at 6 or 18 months or in those with unscheduled deaths. Virus isolation, viral antigen demonstration, and serologic tests for antibodies were not attempted in rats or mice.

With regard to formaldehyde in the exposure chamber, a panel of experts reviewed the method of formaldehyde generation and monitoring and "agreed that the Battelle approach to formaldehyde vapor generation was a suitable adaptation of accepted methods and principles and, therefore, was sound and based upon the best available technology. The same type of assessment applied to the chamber air monitoring system, which also combined two well-established procedures" (*81*).

Suggestive evidence for the carcinogenicity of inhaled formaldehyde vapors also comes from a study by Laskin et al. (*82*). One hundred male Sprague-Dawley rats were exposed to a mixture of gaseous formaldehyde (14.7 ppm) and hydrogen chloride (10.6 ppm) for 6 hr/day, 5 days/week for life. Of the exposed rats, 27% developed nasal tumors, of which 25 were squamous carcinomas and

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*The Chemical Industry Institute of Toxicology recently reported results up to and including the 24 months sacrifice, as follows: in rats exposed at 14.1 ppm there were 93 animals with squamous cell carcinomas, four with squamous papillomas, three with adenomatous polyps and two with carcinomas of the respiratory epithelium; at 5.6 ppm there were two squamous cell carcinomas, four adenomatous polyps and one carcinoma of the respiratory epithelium; and at 2.1 ppm, five rats had developed adenomatous polyps. Additionally, two mice sacrificed at 24 months were found to have nasal squamous cell carcinomas (*80*).

two were papillomas. No nasal tumors were found in 1 0 control rats. Both dosed and control rats had pne monia but the lesions were more severe in dos d rats. The estimated bis(chloromethyl) ether (BC ME) level in the exposure chamber (developing as a result of the chemical interaction of formaldehyd and hydrogen chloride) was 1 ppb. This value was computed on the basis of 10 measurements mac in the mixing chamber during the course of the tudy. It is not certain whether the high nasal can er incidence is a result of the formaldehyde exp sure, the (presumed) BCME exposure, or the biol gical interaction of the two or three compone s of the gas mixture. Based on their previous stu ies (*83*), the authors considered the BCME leve to be too low to be a significant etiological fact r for the nasal cancers observed in this study. They also point to the fact that in rats, BCME caused mostly esthesioneuroepitheliomas rather than squamous carcinomas in the nasal cavities. While the results suggest that formaldehyde (rather than BCME) is the major etiological agent in this experiment, considerable uncertainty remains and other interpretations are possible. The authors' comparison of 20 exposure days at 100 ppb of BCME (*83*) with 500 exposure days at 1 ppb of BCME (*82*) does not, however, resolve the uncertainty of comparing the different factors involved in dose-rate versus total delivered dose in tumor induction.

Three other chronic inhalation studies with formaldehyde designed to investigate possible cocarcinogenic effects of this agent in the upper and lower airways have been reported (*84-86*). Since the nasal tissues were not systematically examined histologically, the value of these studies in assessing the carcinogenicity of formaldehyde is accordingly limited. In spite of these reservations, the studies have some bearing on the problem of formaldehyde carcinogenicity. In the studies conducted at the Oak Ridge National Laboratory (ORNL) by Ne tesheim and colleagues (*84*), Syrian Golden hamste s were exposed by intratracheal (ITr) instillatio to different doses of benz(a)pyrene (BaP) and by inhalation to various concentrations of formalde yde (acrolein exposures were carried out in pa allel studies). Two types of formaldehyde exposu schedules were used: 10 ppm for 5 hr/day, 5 da s/week for life (two groups) and 20 or 50 ppm for 5 h /day, 1 day/week for 17 weeks (seven groups). In ll, nine groups of 88 hamsters each were exposed to ormaldehyde (with or without BaP exposures; tw groups were exposed to formaldehyde only). Significant in the context of this discussion is that no macroscopically recognizable nasal tumors were ob erved in hamsters, whereas in the studies with ra s by Swenberg et al. (*79*) and by Laskin et al. (*82*), grossly visible tumors developed. Based on extensive histopathological evaluation, there was no evidence for the carcinogenicity or cocarcinogenicity of formaldehyde for the larynx, trachea and lower airways. No neoplasms were found in two sections of nose from each hamster, but the level of sectioning was not rigorously controlled.

In the study reported by Horton et al. (*85*), C3H mice were exposed to coal tar aerosol and/or to formaldehyde at concentrations of 40, 80 and 160 ppm. Exposures were carried out for 1 hr/day, 3 days/week for 35 weeks, except for the 160 ppm group which was exposed only for 4 weeks because of toxicity. Only 15 mice survived to 1 year. There is no mention of histopathological evaluation of nasal tissues, so presumably no grossly visible tumors were observed. Coal tar aerosol exposure resulted in lung tumor formation in five animals (one invasive carcinoma) but formaldehyde exposure did not. No evidence was found for any cocarcinogenic effects of formaldehyde. The major shortcomings of this study for evaluating the carcinogenic activity of formaldehyde are that too few animals survived past one year, the individual exposures were short, most groups were exposed only for 35 weeks, and histopathology was inadequately reported.

A third study (as yet unpublished) has been performed by Dalbey and Nettesheim at the Oak Ridge National Laboratory (*86*). In this study, male Syrian Golden hamsters were exposed to diethylnitrosamine (DEN) subcutaneously (0.5 mg once weekly for 10 weeks) and to inhalation of either nitrogen dioxide (10 ppm) or formaldehyde (30 ppm). Exposures to NO_2 and formaldehyde were made once weekly for 48 hr prior to each DEN injection and were subsequently continued for lifetime after DEN injections were completed. An analysis of tumor data indicated that formaldehyde (but not nitrogen dioxide) might act as a tumor "enhancing agent" in the trachea as measured by multiplicity of tumors. This study was limited because, at the doses used, DEN alone produced tumors in a high proportion of animals.

The carcinogenicity of formaldehyde has also been tested by a variety of other routes of administration including subcutaneous injection (in rats), ingestion (by mice and rats), uterine cervical application (in mice) and application to the buccal mucosa (in rabbits). Because of shortcomings in experimental protocols, none of these studies permits firm conclusions regarding formaldehyde carcinogenicity. Nonetheless, some of the studies give definite clues that formaldehyde may be carcinogenic to a variety of target tissues as well as a variety of animal species (and not only to the nasal epithelium of rats).

The most revealing study in this regard is that by Mueller et al. (*87*), who applied a solution of 3% formalin to the oral mucosa of rabbits, using an "oral tank." Each exposure lasted for 90 min and was repeated five times per week for a period of 10 months. As a result, two out of six rabbits developed grossly visible leukoplakias that, according to the authors, showed histological features of carcinoma *in situ*. Unfortunately, the information given on the histomorphology of the lesions is very scanty. Nevertheless, the reported observations appear to be consistent with the rat inhalation studies, suggesting that formaldehyde is a carcinogen.

Also of considerable significance are the observations reported by Brusick et al. (*88*) concerning the transformation of mouse Balb 3T3 cells *in vitro* by formaldehyde concentrations as low as 0.5 μg/ml culture fluid. At this time, no other pertinent information is available on these studies.

Probably the earliest experiments which suggest that formaldehyde is a carcinogen are those by Watanabe et al. (*89*, *90*), who injected rats (strain unknown) subcutaneously with 0.4% formalin (1 ml/week for 15 months) (*89*) and with 9-40% of hexamethylenetetramine (HMT) (from which formaldehyde is liberated *in vivo*) 1-2 ml per week until tumor development (*90*). In the first study (*89*), four of ten rats developed sarcomas at the site of the injection, and in the second study (*90*), eight out of 20 rats developed tumors, seven sarcomas and one adenoma. There is little doubt that tumors were induced by the subcutaneous injections of both chemicals over many months. It is not certain, however, what the role of the repeated severe injury to the subcutaneous tissue may have been in the induction of the sarcomas even through results with formic acid were negative (*90*).

Several other studies carried out with HMT resulted in negative findings. Brendel (*91*) administered HMT daily by gavage to 15 male and 15 female BD rats (0.4 g/day) for 333 days. No treatment-related tumors were observed. Della Porta et al. (*92*) administered HMT in the drinking water to CTM, SWR and C3Hf mice (1.25-12.5 g/kg body weight/day for up to 60 weeks) and to Wistar rats (1.5-2.5 g/kg body weight/day for 104 weeks). No treatment-related tumors were observed. Also negative was a subsequent study in which Wistar rats were given 2% HMT in the drinking water over three consecutive generations (*44*). No evidence for transplacental carcinogenicity was found. It seems clear that, in contrast to long term repeated subcutaneous administration in rats, chronic ingestion of HMT was not carcinogenic to rats and mice. A study by Klenitzky (*93*) in which "formol oil" was applied 50 times to the cervix uteri of mice (strain unknown) resulted in no tumors. It is difficult to interpret this experiment, however, because no information is available on the nature of the material used or on the quantities applied.

Discussion of Laboratory Findings

At present only one study unequivocally demonstrates the carcinogenicity of formaldehyde. This is the study by Swenberg et al. (*79*) which showed a nasal tumor incidence of 20% in rats killed after 18 months exposure to 15 ppm of formaldehyde vapors (the cumulative cancer incidence in the 24-month exposure study is presently not known). No exposure-related tumors have as yet been found in mice which were simultaneously exposed to the same concentrations of formaldehyde. In order to clarify the significance and validity of the CIIT study which, so far at least, appears to be unique in its clear demonstration of formaldehyde carcinogenicity, we will discuss a series of questions which it raises.

(1) Is there any other evidence supporting the results obtained by Swenberg et al. (*79*)? The answer is yes. In the study reported by Laskin et al. (*82*), rats exposed to formaldehyde, hydrogen chloride, and BCME developed nasal squamous cell carcinomas. There is reason to believe that formaldehyde was a major, if not the only, etiological factor (see above). There is evidence that both formalin and HMT (which releases formaldehyde *in vivo*) cause sarcomas in rats when injected repeatedly over long periods of time (*89*, *90*). It has been reported that exposure of the oral mucosa of rabbits to formalin causes advanced preneoplastic lesions (carcinoma *in situ*) (*87*). Formaldehyde has been reported to cause transformation *in vitro* in a mammalian cell line (*88*). Formaldehyde has been shown to be genotoxic in many different test systems (see section on formaldehyde mutagenesis). A closely related aldehyde, namely acetaldehyde, was recently reported to cause nasal cancers (5%) and laryngeal cancers (14%) in hamsters chronically exposed by inhalation to 1650-2500 ppm for 52 weeks (7 hr/day, 5 days/week) (*94*).

(2) Is the carcinogenicity of formaldehyde restricted to only one species, the rat, and only one tissue, the nasal mucosa? Several references cited above suggest that it is not. Species other than rats and tissues other than nasal epithelium seem to be susceptible to the carcinogenic activity of formaldehyde. However, considerable differences in degree of susceptibility may indeed exist. Lesions interpreted to be carcinomas *in situ* were induced in the oral mucosa of rabbits (*87*). Sarcomas were produced by repeated SC injections of formaldehyde

and HMT into rats for many months (*89*, *90*). Neoplastic transformation of mouse Balb/3T3 cells in culture has been demonstrated (*88*). Chronic inhalation of the closely related short chain aliphatic aldehyde, acetaldehyde, has been reported to cause not only nasal but also laryngeal carcinomas in hamsters (*94*).

The nature of the differences in species susceptibility is not clear (such differences exist, however, with almost all carcinogens); they could arise at various levels of organization (physiological, defense mechanism, pharmacokinetics, metabolism, repair capacities). An interesting parallel is the relative resistance of hamsters to BCME as compared to rats (*83*).

(3) Why is the nasal mucosa seemingly the only target tissue in rats for the carcinogenicity of inhaled formaldehyde? Nasal mucosa is directly exposed to formaldehyde and may receive the highest dose although differences in tissue sensitivity and metabolism might also be involved. It should be mentioned that formaldehyde is not the only chemical carcinogen affecting the nasal mucosa. Other carcinogens, in addition to BCME, (including systemic ones) also cause nasal tumors of susceptible hosts, for example, various nitrosamines as well as dioxane and ethylene dibromide (*95*). The experimental induction of nasal tumors in laboratory animals is thus not a rarity and does not necessarily involve direct contact, through inhalation, with the nasal mucosa of the test compound.

(4) Is the viral infection which was noted in the CIIT study likely to be a significant factor in the nasal tumor development of the formaldehyde exposed animals? The possibility that the infection (presumably caused by a corona virus) contributed to the carcinoma response in this study cannot be discounted, but the panel considered it unlikely, because the signs of infection occurred during a very short portion of the experiment (at most a week) and the first detected nasal tumors had probably already formed at the time of the infection. Although the outbreak of sialodacryoadenitis introduced an uncontrolled variable into the experiment, it is unlikely that the transient viral infection affected the outcome of the experiment. It is important to consider that people exposed to formaldehyde may also experience viral infections of the upper respiratory tract.

(5) Are the irritant effects of formaldehyde likely to contribute to its carcinogenicity? "Irritation" is a poorly defined term. As Berenblum pointed out long ago, this term is "a vague generalization representing many different kinds of injury or stimulation of the tissues" (*96*). A more specific effect that has been included under the generic term of "irritation" is the induction of epithelial hyperplasia. Many studies on this topic estimate the degree of irritation by the level of hyperplasia induced in target tissues. The role of irritation in carcinogenesis has been investigated since the early years of chemical carcinogenesis research (*96*).

More recent studies have provided evidence on the hyperplastic effects of several agents, and in some cases have provided qualitative and quantitative analyses of the tissue response. A number of agents were reported to induce epithelial hyperplasia in several types of tissues but they had no carcinogenic or tumor-promoting activity associated with them (*97-99*). The panel found no evidence that the induction of irritation or, more specifically, of epithelial hyperplasia is a sufficient condition for the carcinogenic activity of an agent.

There is, however, suggestive evidence that some agents that produce epithelial hyperplasia may play a role in the process of tumor promotion. It was found that previously initiated mouse skin exposed to a promoting agent for only a short time will develop a low level of tumor response. A high incidence of tumors may develop, however, when the short period of exposure to the promoting agent is in turn followed by exposure to an otherwise inactive irritant such as turpentine (*100*) or wounding (*101*). Repeated mechanical skin abrasion was recently found to have a promoting effect on mouse epidermis (*102*). It is therefore conceivable that the "irritant" effect of formaldehyde, or more specifically its induction of epithelial hyperplasia, may contribute to some extent to the expression of its carcinogenic activity through a mechanism enhancing the promoting or tumor growth stage of carcinogenesis. However, it must be added that our knowledge of this type of effect is still quite inadequate and not directly applicable to the reported carcinogenic effects of formaldehyde on the nasal mucosa.

(6) Are the cytotoxic effects of formaldehyde likely to be an important aspect of its carcinogenic activity? Most carcinogens have significant cytotoxic effects. Therefore, formaldehyde is not an unusual case. There are many reasons why cytotoxic agents which cause cell death and regenerative cell replication might enhance the carcinogenesis process: they increase the number of cells undergoing DNA synthesis [it is known that cells are particularly susceptible to malignant transformation prior to DNA synthesis at the onset of the DNA-synthesis phase of the cell cycle (*102*); they may force the carcinogen exposed target cells through the several cell divisions that seem to be required for "fixation" of the mutagenic or carcinogenic event (*104*); they may create selection pressures in tissues containing initiated cells favoring their proliferation and prop-

agation (*105*); and they may act as tumor promoters (*106*). In short, it is conceivable that the cytotoxic effects of formaldehyde may play a part in its overall carcinogenicity.

Conclusions

In assessing the current evidence for the carcinogenicity of formaldehyde in animals provided by the limited number of studies conducted to date, the panel comes to the following conclusions: (1) The CIIT study provides adequate evidence that inhalation of formaldehyde vapors causes nasal carcinomas in rats. (2) There is suggestive evidence that formaldehyde might be carcinogenic in other species and tissues other than nasal. (3) Formaldehyde should be presumed to pose a carcinogenic risk to humans.

Recommendations for Future Research

The following are recommendations for future research on the carcinogenicity of formaldehyde: (1) the pharmacokinetics of formaldehyde and its interaction with target tissue should be studied in several animal species; (2) studies of formaldehyde by other routes of administration (oral and dermal) should be initiated; (3) additional studies of neoplastic transformation of mammalian cells in culture are needed.

Epidemiology

Strengths and Weaknesses of Epidemiologic Methods

Epidemiologic methods for etiologic studies are designed to uncover disease and exposure relationships in human populations wherein the study groups cannot be experimentally manipulated. Individual studies must be evaluated in terms of the strengths and weaknesses of the study design, the summation of epidemiologic evidence from a variety of studies, however, requires different standards. Circumstances that allow an exposure associated with a disease to be judged a cause of the disease have been best described by Hill (*107*). First is the strength of the association. Second is the consistency of the observation. The evidence for causality is more persuasive when the association has been repeatedly observed under various circumstances by independent investigators using different methods. Third is the specificity of the association. When a specific exposure is associated with a certain disease, there is a strong argument for cause and effect. In light of multicausation of most diseases, however, the lack of specificity does not irrevocably discount a causal hypothesis. Fourth is the temporal relationship of the exposure and disease. Cross-sectional and case control studies are poorly equipped to settle this issue. Fifth is evidence of a dose-response gradient. Sixth is the plausibility and coherence of the hypothesis. The hypothesis should not conflict with generally accepted medical theory.

Completed, ongoing, and proposed epidemiologic studies discussed in this report must be individually evaluated according to the strengths and weaknesses of their design. Conclusions regarding an etiologic role for formaldehyde in disease processes must be drawn while considering findings against Hill's (*107*) points.

Review of Completed Studies

Information on acute and chronic health effects of formaldehyde in humans comes largely from the following two sources: case reports of individuals who were exposed to formaldehyde and cross-sectional studies in which measurements of cause (formaldehyde exposure) and effect (prevalence of symptoms, signs, and disease) were made at the same point in time. In addition, limited information is available from cohort and case-control studies in which the ascertainment of cause and effect relate to two different points in time.

In this report, the assignment of published studies into the above categories was made mainly to facilitate discussion on problems and strengths associated with each category. For the purpose of this report, acute effects were defined as those that develop rapidly after a single exposure, while chronic effects were considered those that do not become manifest immediately after exposure and that persist over a period of time. The acute health effects of formaldehyde have been well documented elsewhere (*108-110*). Therefore, individual studies reporting acute health effects of formaldehyde were not reviewed in detail.

Case Reports

Numerous case reports of health effects, presumably due to formaldehyde, are available. Contact urticaria was described in a 28-year-old woman who worked as a carver and model setter in a factory that made leather dresses containing small amounts of formaldehyde (*111*). She had urticaria almost daily, (severely on the hands, with occasional edema of the lips) during work days. During weekends and vacations, when she had no contact with the leather, there was no evidence of the skin eruption. Similarly, Harris (*112*) described four persons who developed acute papulovesicular eczema following contact with urea-formaldehyde resins. The condition persisted until the workers were reassigned to areas without formaldehyde.

Sakula (*113*) reported a case of acute respiratory distress in a hospital laboratory technician following exposure to formalin. Severe bronchial asthma followed the slightest inhalation of formalin vapor, but the worker was free from attacks on weekends and holidays. A case of pneumonitis following heavy exposure to formaldehyde by inhalation has been reported (*114*). The case involved a 27-year-old neurology resident who spent 15 hr exposed to a high concentration of formaldehyde vapor during preparation of brain specimens for student demonstrations. The following week, after only 2 hr spent at the same activity, he developed acute respiratory distress including progressive dyspnea and chest tightness over a period of 15 hr. Chest x-ray showed increased interstitial markings with early edema. A decreased pulmonary function as measured by FVC, $FEV_{1.0}$ and MMEF was also noted on day 2 and day 24 after the onset of symptoms.* This is said to be the first report describing a clinical picture of acute pneumonitis in man following formalin inhalation.

Cross-Sectional Studies

Acute Effects. Most of the information on the acute health effects of formaldehyde comes from cross-sectional studies of workers, volunteers, and residents exposed to formaldehyde. Symptoms associated with formaldehyde exposure include eye, nose, and throat irritation leading to lacrimation, sneezing, shortness of breath, sleeplessness, tight chest, nausea, and excess phlegm (*115-121*). An outbreak of hemolytic anemia among patients on hemodialysis was described in a recent report (*122*). The outbreak occurred shortly after a new system using filters impregnated with formaldehyde resins was installed. When the filters were removed, hematocrit values returned to previous levels. The severity and incidence of some responses were related to the concentration of exposure. In addition, the extent of reported discomfort appeared dependent on the relative humidity and temperature of the ambient air (*115, 117, 123*).

At 0.1-3 ppm, most people experience an irritation of the eyes, nose, and throat (*115, 117, 123*). Effects of exposures at higher levels are not clear; with increasing concentration, however, the discomfort increases rapidly and many people can tolerate exposures of 4 to 5 ppm for only 10-30 min (*124*). Between 10 and 20 ppm, symptoms are severe, and it becomes difficult to take a normal breath. Exposures at 50-100 ppm and above may cause serious injury to the respiratory tract, such as pulmonary edema and pneumonitis (*124*).

Chronic Health Effects. Chronic health effects attributed to formaldehyde from cross-sectional studies include respiratory problems, dermatitis, neurologic difficulties, and menstrual and reproductive disorders. Schoenberg and Mitchell (*120*) studied five groups of employees from a filter-manufacturing plant to determine adverse effects of exposure to phenolic (phenol-formaldehyde) resin fumes. Groups currently exposed to phenolic resins showed an excess of chronic cough and/or phlegm when compared to previously exposed or "never-on-line" groups. In addition, after adjustments were made for differences in total cigarette consumption, workers on the present production line more than five years had a significantly lower $FEV_{1.0}/FVC$ and MEF 50%/FVC ratio ($p \leq 0.05$) than the "never-on-line" group. These results suggest that long term exposure to phenol-formaldehyde resin fumes may lead to chronic airway obstruction. No systematic measurement of formaldehyde concentration was made during this study but, based on measurements by others, levels were estimated to be in the range of 0.4 to 0.8 ppm. Exceptionally high levels (8.8 to 13.5 ppm) occasionally occurred when cross-current fans were turned off during part of the sampling period. In this study every control subject was occasionally exposed to resin fumes, which may explain the high prevalence of acute symptoms such as eye irritation (80%), nose irritation (53%), and lower respiratory tract symptoms (47%) among "never-on-line" workers. Shift studies suggested a small reduction in lung function in the production line workers, whereas control workers showed a small increase. As noted by the authors, the limitations of this study include small numbers of exposed subjects, probable formaldehyde exposure among some controls, and the potential for selective bias commonly associated with cross-sectional studies. In addition, the possible role of the parent resins, phenol, and other exposure from the industrial process, such as acrylic fiber break-down products, prevents a clear determination that long-term exposure to formaldehyde may lead to chronic airway obstruction.

In a study of rubber workers exposed to hexamethylenetetramine-resorcinol (HR) resins, Gamble et al. (*125*) found more self-reported symptoms

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*FVC = forced vital capacity, $FEV_{1.0}$ = one-second forced expiratory volume, MMEF = maximal mid-expiratory flow rate, same as mean forced expiratory flow during the middle half of the FVC (FEF 25-75%).

(itch, rash, cough, chest tightness, burning eyes, running nose, and persistent cough and phlegm) among HR-exposed than among nonexposed workers. Contrary to the previous findings (*120*), there were no differences in lung function at baseline among exposure groups. There were, however, significant differences in lung function measurements before and after the regular work shift for HR exposed workers, but not among the non-exposed. The resin investigated in this study used resorcinol as the phenol donor and hexamethylenetetramine (HMTA) as both a formaldehyde donor and a catalyst. There was no association between lung function and ambient levels of resorcinol, formaldehyde, hydrogen cyanide or ammonia. Mean concentrations of formaldehyde were 0.05, 0.02 and 0.04 ppm for HR-exposed, HR-not exposed, and control group, respectively. Decrease in pulmonary function was related to the quantity of respirable particulates obtained from personal samples. Chemical analysis of particulates, however, was not performed. It is unlikely that formaldehyde by itself was responsible for the change in pulmonary function for two reasons: (1) the exposure level of formaldehyde was very low and (2) no association between its level and decrease in pulmonary function was observed.

Eight cases of occupational asthma (three smokers and five nonsmokers) were reported among 28 members of the nursing staff at a hemodialysis unit where formalin was used to sterilize the artificial kidney machine (*125*). Recurrent episodes of productive cough accompanied by wheeze were a prominent feature and, for five persons, attacks had extended over the previous three years. Inhalation provocation tests were performed on five subjects with histories of recurrent attacks of wheezing. In two of these subjects the test resulted in asthmatic attacks like those experienced at work. Peak expiratory flow rates fell approximately 50% and wheezing began 2 and 3 hr after exposure to formalin and lasted for 10 hr to 10 days. Three of the five subjects had no respiratory reaction to inhalation of formaldehyde similar to that experienced in the dialysis unit. Two of the five had no symptoms, while one developed conjunctivitis. This latter patient developed redness, weeping and sensations of grittiness of the eyes when heavily exposed to formalin. In the absence of symptoms and exposure there was no apparent reduction in lung function. The authors suggest that although formalin may not have been the etiologic agent in all cases, it may have increased the susceptibility to other agents, which could, perhaps, explain the high incidence of bronchitis-like symptoms. In the absence of a comparison group, an alternative explanation of the asthma being attributable to chance alone still exists. However, the explanation is unlikely because of the high proportion of the staff which developed the symptoms and because of the positive responses observed after the inhalation provocation test.

Formaldehyde-related asthma and dermatitis were also reported by Kerfoot and Mooney (*126*). A survey of six Detroit area funeral homes showed that embalmers were generally exposed to formaldehyde at mean levels ranging from 0.25 to 1.39 ppm with a total range of 0.09-5.26 ppm. They experienced acute toxic effects including eye and nose burning, sneezing, coughing, and headaches. Asthma or sinus problems were reported by [illegible] out of 7 morticians. In addition, two workers experienced dermatitis, with one case being so severe that he discontinued working for a period of time until he recovered. Although it was the professed intention of the authors to correlate the formaldehyde concentrations in each funeral home with the irritant effects on embalmers who worked at the particular funeral home, no data were presented to that end in this study. A dosage effect was implied in the sentence, "The morticians who spent more time embalming than in general funeral work, also experienced more complaints of irritations." It was not clear who and how many people were in the study. Embalming agents contain formaldehyde as well as a variety of other chemicals, such as tissue moisturizers, smooth muscle relaxants, bleaches, an auxiliary antiseptic agent (phenol), dyes, buffers, wetting agents, water conditioners and/or anticoagulants, perfumes and odor suppressors, and vehicles (methanol, ethanol, and glycerine). In light of the possible mixed exposure to a variety of the above chemicals during embalming and the lack of an appropriate control group in the study, the relationship of formaldehyde exposure among embalmers to development of asthma and dermatitis remains inconclusive.

Yefremov (*127*) studied 278 workers at two wood-processing plants where urea-formaldehyde resins were used extensively. They reported that the incidence of chronic diseases affecting the upper respiratory tract was higher in workers exposed to formaldehyde than in a control group consisting of 200 individuals of corresponding ages. The prevalence was from 28.1 to 58.3% depending on work areas, among formaldehyde-exposed workers, compared to 13% among the controls. The highest prevalence of disease (58%) was observed in the workers at the hot press shop, where the concentration of formaldehyde was 2.5 times that in the cold press shop. Disease occurrence was 33.9% at the cold press shop. In all work areas the formaldehyde concentrations were reportedly below the maximum permissible concentration (0.4 ppm). Assuming the age-matched controls represent an appropriate comparison group (no mention was made of matching on sex and smoking history), this report supports the hypothesis that exposure to formaldehyde may lead to chronic respiratory diseases.

Kratochvil (*128*) examined a total of 18 workers of a mean age of 35 with an average formaldehyde exposure of 7 years in a clothing production plant. Formaldehyde exposure occurred during storage and processing of fabric impregnated with urea-formaldehyde and melamine-formaldehyde resins. Catarrhal conjunctivitis was found in 72% of the workers; 28% of the workers exhibited a catarrhal inflammation of the upper respiratory tract, 22% of the workers exhibited chronic bronchitis diagnosed in accordance with the criteria of the World Health Organization, and 11% of the workers showed skin changes. Lung function tests showed that the vital capacity and the differential forced vital capacity exhibited normal values for all workers. The concentrations of formaldehyde in these work areas were below the maximum allowable concentration (0.4 ppm). In the absence of an appropriate control group, an etiologic role of formaldehyde in development of the above conditions is uncertain. In fact, the 22% incidence of chronic bronchitis among the workers corresponded to the incidence of this condition reported for a normal population of a similar age group.

Shumilina (*129*) reported a high incidence of menstrual and reproductive function disorders among 446 women workers (130 finishers and 316 inspectors) exposed to urea-formaldehyde resins. Formaldehyde concentrations of 1.2 to 3.6 ppm were often found in the finishers' work area of the fabric trim shop, while levels from 0.04 to 0.56 ppm occurred in the inspectors' work area. A group of 200 saleswomen not exposed to formaldehyde was used for comparison. The reproductive disorders reported to be more common among those exposed included menstrual disorders, increased complication during pregnancy, and higher percentage of neonates with low birth weights. The role of formaldehyde in the development of these disorders is uncertain, however, because of the lack of information on the work environment and the socioeconomic status of the study and control groups. In addition, many of these disorders are known to be associated with physical and mental stress, personal habits (alcohol, cigarettes, caffeine consumption), nutritional status, and other factors related to the socioeconomic status of women. Still, these findings are intriguing and indicate a need for further studies in this area.

Neshkov and Nosko (*130*) reported a high incidence of sexual dysfunction among male workers employed in a plant producing glass fiber reinforced plastic. These workers were exposed to vapors of phenol, formaldehyde, aniline, epichlorohydrin, diphenylpropyl, styrene and a combination of glass fiber and glass-reinforced plastic dust. The levels of each of these chemicals were 1.5 times the respective maximum permissible level in 63% of the samples (maximum permissible level of formaldehyde is believed to be 0.5 mg/m^3 or 0.4 ppm). Among the 143 workers examined, 58 (40.5%) had psychoneurologic and sexual complaints. Sexual complaints included a diminution of libido, premature ejaculation, a weakening erection and decrease in the satisfaction derived from an orgasm. Analysis of the sexual complaints revealed a direct relationship to the duration of employment at the plant. Testicular dysfunction among the workers was also reported, including decreased volume and increased viscosity of ejaculate and decreased number of spermatozoids. The authors concluded that these sexual dysfunctions were the results of complex toxic effects of chemicals on the cells of the cortex and those of the subcortical brain stem structures, which participate in the regulation of sexual function. It is impossible to determine how much of the sexual dysfunction might be attributable to formaldehyde since appropriate control groups were not included and these workers were exposed to a variety of toxic chemicals in addition to formaldehyde. Of particular interest are reports of epichlorohydrin-induced sterility in animals (*131*, *132*).

Ishchenko and Pushkina (*133*) compared morbidity and sick leave among 662 workers engaged in the manufacture of phenol-formaldehyde resins to that among 473 unexposed workers. Several disease conditions were more common in the formaldehyde exposed group than in the controls, particularly from diseases of the respiratory tract (1.8-fold among males and 1.4-fold among females). Among women, the frequency of urogenital problems was 2.3 times more common in the exposure group. Days absent from work due to illness among those exposed exceeded controls 1.5- to 1.6-fold. Although the authors state that the age distribution of the exposed and comparison populations was similar, no description of the methods used to determine morbidity (examination, self-report, or medical records) was provided. In addition, uncontrolled social factors, as recognized by the authors, may play a role. In light of these limitations, the greater number of lost workdays due to sickness and the increased evidence of respiratory and urogenital diseases among the exposed workers, although suggestive, does not provide definitive evidence concerning the role of formaldehyde.

In a mail survey of 20 funeral homes in Los Angeles (*134*), 57 of 80 embalmers responded. Nine (16%) reported symptoms compatible with acute bronchitis and 17 (30%) were considered to have chronic bronchitis. The 31 asymptomatics, however, had worked longer than the bronchitics (18 years vs. 11 years). In the absence of a control group, these findings are, at best, suggestive.

Engel and Calnan (*135*) reported an outbreak of dermatitis in a car factory. A total of 50 cases of dermatitis were observed in three years (1962-1965) among 150 employees who handled rubber weather strips coated with phenol-formaldehyde resins. The workers who developed dermatitis had been exposed to the adhesives containing phenol-formaldehyde resins for from one day to two years before the onset of the eruption, with an average period of contact of 17 weeks. The average duration of the eruption was 12 weeks, however, in three cases it persisted up to 2½ years. The eruption was generally an erythematous vesicular rash of the fingers and hands. Three materials were handled by these employees: (1) the rubber weatherstrips, used for some years; (2) adhesive A and B introduced 4 years before in 1962 and supplied by the same manufacturers; (3) toluene, which was the solvent used to activate the adhesive. The rubber weatherstrips alone were ruled out as a cause because they came from various suppliers and had not changed in composition for a long time. The toluene would not be expected to cause sensitization within a short period of time. Among the 29 patch-tested dermatitis patients, four (14%) gave a weak reaction to phenol alone, while 65% had a positive reaction to the adhesive resins. It is, therefore, probable that formaldehyde in the resins was a causal agent. Outbreaks of dermatitis in several industries using formaldehyde resins were reported by Schwartz, Peck and Dunn (*136*). In a factory where plywood was laminated, 600 cases of dermatitis were reported among about 800 workers during the first six months of operation. In a second reported outbreak, over 40 workers out of a total of 100, developed dermatitis in a factory making tool handles from laminated glass fabric and phenol-formaldehyde resins. Although no unexposed group was available for comparison, the high proportion of formaldehyde exposed workers developing dermatitis is quite impressive.

In a hemodialysis unit where formalin was used as a sterilant, 6 to 13 staff members developed dermatitis within 3 weeks (*137*). Four of the six were positive in patch tests with 3% formalin. It was not clear why only the hemodialysis unit was affected, since other units also used formalin. The author speculated it might be due to the use of a detergent that lowered the resistance of the skin to formaldehyde vapors and to the high temperature and concentration of formalin in the preparation room. Lack of controls hampers the interpretation of these data.

Cohort and Case-Control Studies

The panel was not aware of any published cohort of case-control studies designed to evaluate the role of formaldehyde in the development of disease or abnormal conditions. Many mortality studies have been reported of workers from occupations or industries wherein formaldehyde exposure may have occurred. For example, Moss and Lee (*138*) have reported elevated risks of oral and pharyngeal cancer among male textile workers. They reported 77% excess deaths due to these cancers compared with the male population of Wales and England. These textile workers may have been exposed to formaldehyde, since formaldehyde has been widely used in the textile industry for producing creaseproof, crushproof, and shrinkproof fabrics. No environmental measurements of formaldehyde, however, were made in this study. A recent NIOSH survey showed concentrations of formaldehyde in the textile plants in the U.S. ranging from 0.1 to 1.4 ppm.

Bross et al. (*139*) reported a significantly increased risk of nasal cancer among brickmasons, textile workers and shoemakers. Textile workers also had significantly elevated risk of cancer of the pancreas and stomach. No information on formaldehyde concentrations was reported.

Decoufle (*140*) has reported significantly increased risks of cancer of the buccal cavity, pharynx and larynx among male leather workers. Bladder cancer and malignant lymphomas were also associated with increased risks among male and female employees in the leather industry. The cancer risk for the employees was calculated relative to the risk for those who were in clerical positions. A variety of chemicals including formaldehyde, azo dyes, chromium compounds and tanning extracts have been used in the production of leather goods. Some of these chemicals are carcinogenic in animals. Industrial hygiene surveys of a calf skin tannery in the U.S. showed concentrations of formaldehyde in the finishing department ranging from 1 to 8.6 ppm (*141*).

Matanoski (*142*) reported an excess of primary liver cancer and lung cancer among pathologists compared to radiologists. However, oral and pharyngeal cancers were lower in the pathology group. Although exposure estimates were not available, pathologists are more likely than radiologists to be exposed to formaldehyde.

Although the above investigations were not designed to study health effects of formaldehyde, the results suggest the possibility of formaldehyde being carcinogenic in humans. The lack of precise information concerning the number of persons exposed to formaldehyde and the level of exposure, in addition to the problems of confounding exposures, makes interpretation of these reports regarding formaldehyde difficult.

Conclusions

In summary, a wide variety of acute and chronic health effects from formaldehyde exposure have been reported. They include eye, nose and throat irritation, headache; shortness of breath; wheezing; dermatitis; chronic cough; excess phlegm; chronic airway obstruction; asthma; bronchitis; rhinitis; pharyngitis; menstrual and reproductive disorders; sexual dysfunction; and possibly cancer. These studies were primarily of a cross-sectional design, thus, they do not allow a clear determination of the order of events. In addition, many studies lacked appropriate controls and/or environmental measurement, making it difficult to clearly evaluate the role of formaldehyde. Independent reports of respiratory system disorders and dermatitis among persons from a variety of work settings supply evidence on the consistency of the association, and indicate that formaldehyde is a likely contributor to the origin of these diseases. Only three studies (*120, 125, 127*) allowed an estimate of the strength of association between formaldehyde and respiratory disease. The risk of chronic respiratory symptoms among exposed groups ranged from about 1.5 to 4.5 times that among nonexposed groups, with little evidence of a dose gradient. The studies of dermatitis among workers exposed to formaldehyde, although reporting a high prevalence, lacked comparison populations necessary to estimate the strength of the association. Respiratory and integumentary systems are plausible response sites since workplace exposure to formaldehyde is likely to occur by inhalation or dermal contact.

The report of excessive menstrual and reproductive problems among women exposed to formaldehyde is provocative and, despite the study limitations, indicates the need for further research in this area. No reports of studies specifically designed to evaluate the carcinogenicity of formaldehyde in human populations were available, but several such studies are in progress.

Review of Planned and On-Going Epidemiological Studies of Formaldehyde

Planned and on-going investigations related to formaldehyde exposure include one study of a new technique of measuring formaldehyde, three studies investigating complaints, four epidemiological studies of morbidity (cross-sectional and prospective), and six mortality studies (cohort, proportionate mortality, and case-control). The questions being addressed and an evaluation of each group of studies follows.

Measurement Technique

The use of solid absorbent surfaces for collecting personal samples of vapor and particulate formaldehyde is being examined. This method should be more sensitive and efficient than the present impinger method, thereby permitting more accurate assessment of environmental exposures. An occupational study (cross-sectional morbidity) is planned, but details have not yet been provided.

Investigation of Complaints Relating to Formaldehyde

The results from three states are being compiled. In Connecticut, persons complaining of health problems due to urea-formaldehyde insulation completed a questionnaire concerning their symptoms and air levels of formaldehyde were measured in their homes. Occupants 489/794 of 262 residences presented complaints, the most common symptoms being eye, nose, and throat irritation, and headaches. Formaldehyde concentrations ranged from 0 to 10 ppm (mean of 1.5 ppm). About half of the complaints were associated with levels of 0.5 to 10 ppm.

F. Marshall of the New Jersey Department of Health obtained medical histories, all available information on insulation, and patterns of health effects from residents with recently installed urea-formaldehyde foam insulation who complained of formaldehyde odor, irritation, or increased pre-existing illness patterns. In 40/55 homes investigated, the most common symptoms were tearing of the eyes, sore throat, cough, and runny nose. Air samples were collected in 22 homes. In 14 homes where formaldehyde was detected, levels ranged from 0.01 to 0.78 ppm.

In New Hampshire, the Bureau of Occupational Health (M. Hilgemeier) administered a standard questionnaire to residents with complaints associated with formaldehyde exposure. Standard NIOSH techniques were used to collect formaldehyde samples. About 90% of the samples were below 0.5 ppm; 10/77 dwellings were mobile homes.

The above studies on formaldehyde complaints are all similar and suffer the same deficiencies. None of them can be used to estimate prevalence of symptoms nor can they be used to establish dose-response relations. These shortcomings include: (1) the surveys are conducted only among those who complain, and although the prevalence of symptoms should be high, it is a biased sample; (2) there are no controls and no measurement of other irritants that could cause health complaints. Thus, these studies are only suggestive of possible problems and by themselves provide little evidence of cause and effect relationships.

Morbidity

R. Levine of the Chemical Industry Institute of Toxicology studied approximately 100 West Virginia morticians in a cross-sectional study of lung function (spirometry) and respiratory symptoms. Employment and smoking histories were also obtained. Industrial hygiene measurements made in the mortality study of Ontario embalmers along with symptoms during embalming, number of bodies embalmed, subjective assessment of severity of fumes, and length of employment will be used to assist in the estimation of exposure in this study. Spirometry will be compared to predicted values from Knudson, and internal comparisons will be related to exposure after age, height, and smoking adjustments. Data from this study are being analyzed in terms of the prevalence of respiratory symptoms (cough, phlegm, dyspnea) and the relation of percentage of predicted pulmonary function to employment (formaldehyde exposure) after adjustment for confounding variables. There is no assessment of skin effects. It is not clear if acute symptoms related to exposure are to be evaluated. Although there is a good rationale for studying this occupational group, an inability to detect a significant response in this study could be due to low cumulative exposure (probably less than 8 hr/day). Length of exposure (years worked or latency) should be adequate. If we assume 50 persons each (smoking groups combined) in a high and low exposure group (the optimal situation) there is an 85% chance of correctly rejecting the null hypothesis of no difference in FEV between exposure groups, if the true difference is 0.5 liters and α level is 5%. If the symptom rate is 20% in the low exposure group, there is less than a 50% chance of correctly rejecting the hypothesis of no difference if the true difference is 20% at the 5% level (two-tailed test). As in all cross-sectional studies, estimates of exposure are only a crude indicator of actual exposure. This is, however, one of the few studies to attempt to assess long term effects of formaldehyde on lung function. Although embalming fluid contains 2% formaldehyde, other pulmonary irritants are also present (e.g., phenol) but these may be at low enough concentrations to have a minimal effect. A comparison group (e.g., funeral directors) would have provided more confidence in the results and more power in the analysis.

The remaining three morbidity studies are of residents in homes with urea-formaldehyde (UF) foam insulation. In the first of these, M. Thun of the New Jersey Department of Health identified about 400 homes that had been insulated with UF foam and an equal number of control homes. Information gathered by telephone interview included type of home and insulation, formaldehyde odor, health symptoms and other medical data, demographic data on the occupants, and the period prevalence of asthmatic attacks, wheezing, chest pain, stinging or burning skin, burning or tearing eyes. Except for wheezing, the controls reported a higher prevalence of symptoms than did UF foam households. There was no detectable difference in the overall incidence of new symptoms during the year. There was, however, an association of increased symptoms and formaldehyde odor; the incidence of new symptoms following installation of the insulation was 2.7 times higher than in the months preceding the insulation. About 64% of the residents of the UF foam homes reported no problems at all related to the foam insulation. There is the possibility of selective recall in the remaining 36% of the study group because of the possible publicity relating to mobile homes and the specific questions relating to insulation. Despite this potential bias, there was little overall difference between study and comparison populations. There was, however, a dose-response relation (dose was based on odor). Unfortunately, there were no environmental measurements to correlate with odor perception and symptoms, and apparently no control of other potentially confounding irritants other than formaldehyde. The added insulation may have also increased other respiratory irritants that could have raised the symptom rate. It is not clear how the formaldehyde odor was described to the subjects. This study has been completed recently and should be available for review shortly. The summary given the Panel suggests little difference between the insulated and control homes except when odor occurred.

M. Woodbury and C. Zenz of the Wisconsin Department of Health and Social Studies are proposing to study 110 new mobile home owners. Exposure measurements are to be made twice monthly for 9 months. It is difficult to evaluate this prospective study because of the lack of detail provided. Some of the questions that need answering are: What are the health parameters that will be determined and how often are they being monitored? Are there formaldehyde controls for the mobile homes? If so, how are they selected? If no controls are planned, the study will have limited value. Are other respiratory irritants being measured (e.g., NO_2)? About 50% of the homes are newer homes with higher formaldehyde levels than the older homes which are serving as controls and have measurable but low levels of formaldehyde. Assuming a 20% symptom rate and 180 people in each exposure group (high and low formaldehyde), there is about an 85% chance of correctly rejecting the hypothesis of no differences in symptoms when the true difference is 15% at the 5% α level, and about a 99% chance of correctly rejecting the hypothesis of no difference in FEV_1 when the true difference is 500 mL at the 50% α level (two-tailed test). The effects of long-term chronic exposure cannot be estimated by this study. These investigators also performed a retrospective pilot study of 65 mobile home residents. The data will be mainly useful in the planning and execution of the prospective study.

L. Williams of the Oregon Department of Disease Monitoring and Control is planning to study 300 mobile home residents who responded to a health questionnaire. The volunteers were from two regions, coastal and inland. The purpose of the study is to ascertain the effect of humidity, temperature, and wind ventilation on "health effects to potential formaldehyde exposure." Inasmuch as the participants in the study are volunteers, however, it is not a random sample. Apparently, environmental measures are planned, but it is not known what other information on insulation, demography, other pulmonary irritants, humidity, temperature, etc., will be collected. Although the details are unknown, this study design does not promise to add very much to our knowledge. The power in this study is similar to the study by Woodbury and Zenz when the following assumptions are made: (l) the two exposure groups to low and high formaldehyde are of equal size (~ 150); (2) the prevalence of symptoms is about 30% in low exposure group.

Mortality

H. Weiss of the Wisconsin Department of Health and Social Sciences conducted a case-control study of infant mortality by type of residence and found an increased risk for mobile home residents. Socioeconomic status is only one of many sources of potential bias in this study. The completed study should be reviewed, but the short description available suggests that the data will provide little, if any, useful information on the effect of formaldehyde.

D. Grauman of the National Cancer Institute proposes to evaluate the mortality experience (using standardized mortality ratios [SMR] of a cohort of about 11,000 medical technologists. This group is exposed to other chemicals in addition to formaldehyde (e.g., chloroform and benzidine). No environmental data are available, so there cannot be a good estimate of exposure-response relationships. A positive association will not be conclusive because of the mixed exposure. A negative association will not be conclusive because of the lack of exposure information and the possibility of low exposures. The control group is to be the U.S. population. If 15% of the cohort are dead (n = 1650 cases) and if 20% of the deaths in the control population were from cancer, there would be better than a 95% chance of correctly rejecting the hypothesis of no difference between exposed and controls if the true differences were 5% at the 5% α level. Assuming 1/4 of all cancers were lung cancer, there is about an 80% chance of correctly rejecting the hypothesis of no difference if the true differences were 5% at the 10% α level (two-tailed test).

There are three mortality studies of embalmers. J. Walrath is conducting a proportionate mortality ratio (PMR) analysis of about 1500 morticians from New York. The purpose is to determine whether there is an excess proportion of deaths due to specific malignant neoplasms compared to the general population. Besides having the problems inherent in a PMR analysis where the population-at-risk is not available there is little exposure data (length of exposure is estimated on the basis of the year first licensed). Assuming the rate of lung cancer is 5%, there is better than a 99% chance of correctly rejecting the hypothesis of no difference in lung cancer rates when the true difference is 5% at the 1% α level (two-tailed test). Walrath is also conducting another PMR study on about 1200 embalmers from California. This study is very similar to the one in New York, except there will be more information on length of exposure which can be evaluated. There is, however, no direct estimate of exposure or smoking history or other confounding exposures. If 20% of the deaths were from cancer, there would be a better than 90% chance of correctly rejecting the hypothesis of no difference in cancer rates between exposed and controls if the true differences were 5% at the 10% α level (two-tailed test). R. Levine of the Chemical Industry Institute of Toxicology is also studying embalmers using an SMR retrospective mortality design. The cohort is composed of Ontario's funeral service professionals licensed during 1914-1967. Date of birth, date of first licensure, type of license, years licensed, and place of employment are available for each person. Observed mortality rates will be compared to Canadian national and provincial mortality data. Mortality will also be analyzed as a function of exposure (years worked?). A retrospective industrial hygiene assessment will consist of inquiring about changes in funeral practices with time, surveying several selected funeral establishments. The survey will include air sampling for agents of potential health concern (e.g., formaldehyde, phenol) and examination of purchase records to determine amount and kind of chemicals used. Presumably these data are to be used in evaluating dose-response relationships. Assuming the study population comprises 180 deaths (15% of the estimated cohort) and a 20% cancer rate there is about a 50% chance of correctly rejecting the hypothesis of no difference if the true difference is 10% at 5% α level (or an 85% chance if the true difference is 15% at the 5% α level). As a group, these three studies of embalmers should provide a good estimate of potential risk from formaldehyde exposure.

A. Blair at NCI and the Formaldehyde Institute are collaborating on a proposed cohort mortality study to develop age, race, and sex-specific mortality rates among formaldehyde-exposed workers. Rates will be compared with those in the U.S. population and local or regional populations where appropriate.

The first phase of the investigation is to determine if there is a suitable cohort for study. Suitability will be based on representatives of the participating companies, range of exposure, availability of a cohort of at least 3,000 workers with a minimum 15-year latent period, suitable age distribution, and sufficient information for adequate follow-up. If the requirements for a scientifically sound study are met, the study will proceed. Formaldehyde exposure for each individual will be estimated using job titles, work locations, past environmental measurements, years of employment, and the presence of potentially confounding exposures. The exposed group will be stratified by intensity and duration of exposure, age and year of first exposure, susceptibility, and latency.

The cohort should be large enough to engender confidence in the differences in mortality experience of study and comparison populations. Although exposures are unlikely to be pure formaldehyde, because of the varied occupational exposures and size of the cohort, it may be possible to assess the individual contribution of formaldehyde. Although the assessment of exposure is retrospective, it can still be at least semiquantitative. This is a very important part of this study and should employ a full-time industrial hygienist following methods similar to those of Emsden at the University of Pittsburgh. It is important that all companies participate in the study, as the results may be seriously biased if some do not.

Conclusions

Of all the mortality studies proposed, those examining the medical technologists, embalmers, and formaldehyde workers are the only ones that can assess the carcinogenic risk among those individuals exposed. Although single epidemiologic studies usually cannot adequately assess an occupational risk, or the risk of exposure to a chemical agent, taken together these studies should help clarify the situation. There are, however, a number of concerns about these studies.

- Are exposures to formaldehyde high enough in these populations to assess possible effects? For example, are embalmers exposed 8 hours/day, 5 days/week, or is their exposure much less?
- Can exposure history be adequately documented? Are individuals with little or no embalming experience distinguishable from those with considerable exposure? Since few if any of the workers are exposed only to formaldehyde, what is the effect of exposure to these other agents?
- Is exposure to formaldehyde in the same range as for other populations?

The confidence we place in the findings from these studies depends largely on the ability to retrospectively estimate exposure. If exposure in these occupational groups is representative of other exposed populations, the conclusions may be indicative of potential risk to all exposed populations. If these studies do not adequately address the above questions, then further investigation may be required. Such investigation may be pursued within the existing data set, or new populations may need to be investigated.

Recommendations for Epidemiologic Research

There is a need for carefully designed epidemiologic studies to evaluate the role formaldehyde may play in the origin of certain chronic diseases. Specific research areas identified by the panel include the following: (1) additional studies of chronic respiratory system disorders that include environmental measurements to allow a more precise estimate of risk at various exposure levels, including industrial, as well as mobile home populations; (2) projects designed to confirm or deny the association of menstrual and reproductive disorders and formaldehyde exposure reported by Shumilina (*45*); (3) more complete epidemiologic studies to evaluate the carcinogenicity of formaldehyde in human populations. Although several projects are under way, there is a need to be alert for resources for additional research. The widespread use of formaldehyde in industry and its occurrence in a variety of consumer products may provide opportunities for other studies. The many uses of formaldehyde are summarized in the NIOSH Criteria Document (*108*) and will not be itemized here. It is sufficient to say that the chemical is of importance in adhesives for particle board and plywood production; resins to mold a variety of plastic parts for automobiles, appliances and hardware; wrinkle-resistance in textile manufacturing; strengthening of various paper products (grocery bags, wax paper, napkins and tissues, and filter paper); specimen preservation; mildew prevention; insulation; and protective coatings.

It may be possible to identify other formaldehyde-exposed cohorts for study. The major difficulty is that, for most occupations or industries, only a small proportion of the workers have actual contact with formaldehyde. This is clearly demonstrated from the NIOSH Occupational Hazard Survey, Phase I, 1972-1974, where 4,636 plants were studied in over 600 different industry types (according to S.I.C. codes). These plants employed 895,725 workers in 453 different occupations. Products containing formaldehyde were encountered in 396 separate S.I.C.-coded industries. Table 1 lists the percentage of workers exposed to formaldehyde in the industries with the larger work forces, and those of particular interest.

This survey confirms an impression given by published reports that exposure to formaldehyde is more common in medical and laboratory environments and in certain parts of the textile, wood, and paper industries. In certain industries, however, the number of work places and workers studied was small and the survey may not have included those where significant exposure to formaldehyde occurs (e.g., the chemical manufacturing industry).

Table 1. Percentage of workers exposed to formaldehyde.

Industry	% of workers exposed
Medical	
Veterinarian and animal hospitals	23.0
Funeral services	100.0
Medical laboratories	31.0
Construction	
Forestry services	10.5
General building contractors	3.6
Heavy construction	3.7
Plastering and lathing	5.4
Textiles	
Finishing plants (synthetics)	11.6
Coated fabrics	14.0
Hats and caps	47.0
Fabricated textile products	3.3
Wood and paper	
Veneer and plywood	21.0
Wood products	8.6
Upholstered furniture	12.2
Paper mills	5.8
Paper coating and glazes	9.0
Bags (except textiles)	11.1
Paints and allied products	16.9
Fabricated rubber products	29.0
Abrasive products	11.0

The Industry-wide Studies Branch of NIOSH is carrying out an industrial hygiene study of formaldehyde exposure in several industries in search of a suitable cohort for a mortality study of formaldehyde exposed workers. Measurements will be made in industries concerned with formaldehyde manufacture, textile and clothing production, wood furniture and wood and paper product manufacture. Fifteen site visits are planned and these will be completed over the next six months.

The major difficulty of the mortality studies of individuals exposed to formaldehyde is the limited ability of such studies to detect excess risk for rare causes of death. Since the known carcinogenic action of formaldehyde is limited to the nasal sinuses in rats, there is a need to evaluate the risk for this site in man, although it is clear that carcinogens may not affect the same tissues in humans as in laboratory animals. It is unlikely that a cohort of sufficient size can be assembled to accomplish this task; however, a carefully designed case-control study might. The panel suggests that the feasibility of performing a case-control study of cancer of the nasal sinuses in areas where there is heavy production or use of formaldehyde as a vapor should be explored.

Evaluation of Risk

Nasal Tumor Risk in Rats Due to Formaldehyde Inhalation

Existing data on humans exposed to formaldehyde provide no quantitative estimates of the incidence of irreversible diseases. Epidemiologic studies are in progress which may be useful in identifying and estimating chronic disease risk due to formaldehyde vapor exposures.

A study was sponsored by the Chemical Industry Institute of Toxicology to investigate the toxic effects of inhalation of formaldehyde vapor in Fisher 344 rats and B6C3F1 mice. Animals were exposed to average dosages of formaldehyde vapor of 0, 2.1, 5.6 and 14.1 ppm for 6 hr/day, 5 days/week, for 24 months. A total of 120 animals/species/sex were exposed at each dosage level. Within each group, 10 animals were sacrificed at 6 months, 10 animals at 12 months and 20 animals at 18 months. The frequency of nasal tumors in Fischer 344 rats through the 18 month sacrifice was reported by Swenberg et al. (*79*). Since no significant differences were noted in the frequency of nasal tumors between sexes during the first 18 months of exposure, the results for the sexes were combined. No nasal tumors were observed prior to 12 months. The results given are summarized in Tables 2 and 3.

Tumors in two categories were considered for risk analysis: squamous cell carcinoma and total nasal tumors. Since time-to-tumor data were not presented by the authors risk analyses are performed only on the unadjusted tumor rates of animals dying with tumors prior to 18 months. The number of unscheduled sacrifice animals at risk for the combined sexes prior to 18 months is 200 per dose.

Using a procedure given by Crump et al. (*143*), the multistage model of carcinogenesis was fit to the proportion of animals dying with tumors before 18 months. Note that this analysis does not give the probability of death due to tumors. Time-to-tumor and cause of death data are not given by Swenberg et al. (*79*); hence a competing risk analysis utilizing the sacrificed animals cannot be performed. The upper 95% confidence limits for the proportion of animals dying with squamous cell carcinomas before 18 months were 0.3, 2.0 and 17.1% for 2.1, 5.6 and 14.1 ppm, respectively. Even though no squamous cell carcinoma were observed at the two lower dosages, because of statistical uncertainty there is no guarantee that squamous cell carcinomas cannot occur at 5.6 ppm or less in Fischer 344 rats within 18 months.

Since the true form of the dose response curve below 2.1 ppm is unknown, nasal tumor rates in rats exposed below this level cannot be predicted directly. Since the dose response is curving upward (convex), the potential nasal tumor rates at lower dosages are predicted to be proportionately lower than the potential risk at 2.1 ppm. The upper confidence limit on the rate of squamous cell carcinomas is 0.3% at 2.1 ppm. At 1 ppm the risk is predicted to be at least a factor of 2.1 lower, i.e., 0.003/2.1 = 0.0014, etc. In general, proportional dose response predictions (linear extrapolation) for dosages below 2.1 ppm are given by: Squamous cell carcinoma rate ≤ 0.0014 × dosage (ppm).

Similarly, the upper 95% confidence limit for the proportion of animals dying before 18 months with

Table 2. Frequency of nasal tumors in animals dying prior to 18 months.

Dosage, ppm	Number dying	Tumor frequency		
		Squamous papilloma	Squamous cell carcinoma	Spindle cell sarcoma
0	2	0	0	0
2.1	2	0	0	0
5.6	8	0	0	0
14.1	44	4	28	1

Table 3. Frequency of nasal tumors in animals sacrified at 18 months.

Dosage, ppm	Number examined	Tumor frequency	
		Adenomatous polyp	Squamous cell carcinoma
0	40	0	0
2.1	40	1	0
5.6	40	1	0
14.1	40	1	8

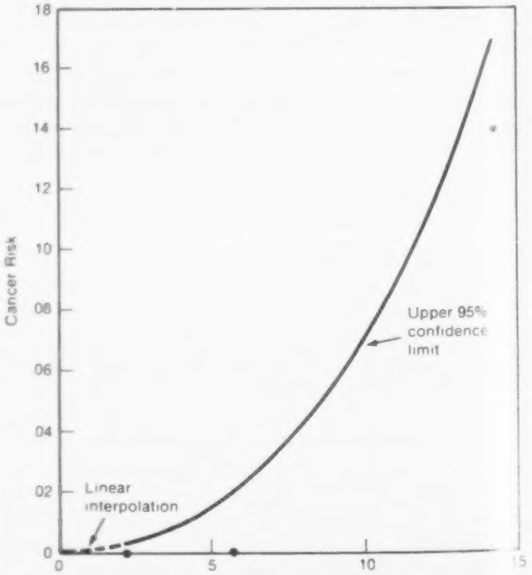

FIGURE 4. Potential proportion of Fischer 344 rats dying with squamous cell nasal carcinomas before 18 months.

any nasal tumor is 0.33%, giving: Total nasal tumor rate $\leq 0.0016 \times$ dosage (ppm). For example, the proportion of animals dying before 18 months with squamous cell carcinomas in Fischer 344 rats is predicted to be no more than 1.4 per 1000 animals at an exposure of 1 ppm formaldehyde for 6 hr/day, 5 days/week. The low dose extrapolation procedure is illustrated in Figure 4.

The rat data indicates a potentially high nasal tumor rate at inhalation exposure levels comparable to levels which can be experienced in homes and occupational environments. Perhaps the epidemiology studies in progress will provide direct tumor risk information for humans exposed to formaldehyde.

Conclusions

The metabolic pathways for formaldehyde in various animals species and man are qualitatively the same but differ in rate with formaldehyde or a formaldehyde adduct likely to be the carcinogenic agent.

Most of the studies available on the possible teratogenicity or reproductive effects of formaldehyde are inadequate for evaluation and none are adequate for exposure by inhalation.

Formaldehyde is mutagenic in a variety of test systems, including bacteria, yeasts, fungi, insects and mammalian cells and causes mutations and chromosome aberrations.

By inhalation formaldehyde is carcinogenic to the Fischer 344 rat, producing nasal tumors at dose levels that are within the same order of magnitude as those to which humans are exposed.

Formaldehyde may be carcinogenic to species other than the rat and to tissues other than nasal.

Formaldehyde should be presumed to pose a carcinogenic risk to humans.

No information is presently available whether (a) certain individuals may be at greater risk of cancer from formaldehyde exposure, (b) tumors may be expected to arise at nonrespiratory sites as a result of inhalation of formaldehyde by humans, or (c) the effects of formaldehyde exposure may contribute in an additive or synergistic way to the effects of other carcinogens or tumor promoters.

We thank Ms. Eva S. Lehman, CPSC, for her assistance in the preparation of the manuscript.

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Vol. 43, p. 169, 1982

Letter to the Editor

A recent letter (Saxenmeyer, Environ. Health Perspect. 37:202) concerned the safety of nuclear waste disposal, which is a very real and important problem. The comments here should not be construed to indicate that I favor all types of nuclear waste disposal, but to indicate that despite the high intentions in the letter, it appeared to be below EHP standards and portions were inappropriate.

First, many of the points and comparisons made were weak or illogical. The lifetime of a motor or engine, for example, doesn't provide information about the stability of geologic formations. Why must we settle for a disposal process lasting up to 200 years, or none at all, and how is 200 years arrived at? There is no possibility of extrapolating in all details to future time from a "critical analysis of the age of the earth."

Second, even if the earth were only 100 years old, we would already know about massive earthquakes, volcanism, flooding and such. It is the calibration of time that varies, not the absolute number of years. Using the premise of a 6000-year-old earth doesn't affect our knowledge of earth and atmospheric instabilities any more than does a 4.5 billion-year-old earth.

And third, at least in this instance it was inappropriate for ENVIRONMENTAL HEALTH PERSPECTIVES to be a vehicle for unnecessary controversy concerning age of the earth. There is already a field that could just as well be called "scientific creationism" if the term "creationism" hadn't obtained the connotation it has. Most of the physical and biological data do not support anything near a literal, 6000 year history of earth based upon the Gregorian improvements of the Julian calendar used in most of the western world.

Confusion stems from insufficient information and from semantics. Science doesn't operate to validate previous beliefs but to learn, eventually, what is closest to coherence over a large variety of frameworks, truths as closely as these can be approached. The scientific bases of geologic principles, age of the earth and evolution are broad and substantial. Outright disregard or improper distortions tend to reduce the clarity and credibility of the positions taken.

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Environmental Health Perspectives
Vol. 43, p. 171, 1982

Announcement

Dioxin 82

3rd International Symposium/Workshop on Chlorinated Dioxins and Related Compounds

The aim of this symposium to be held at Salzburg, Austria on October 12-14, 1982, is to bring together specialists from different fields of dioxin research, which will allow the problem to be treated in an interdisciplinary fashion. Similar to the first successful workshop in Rome and the second event in Washington, October 1981, emphasis will be on discussion and exchange of information. Symposium Proceedings will be published.

Topics will include incineration sources, analysis and standards, fate, distribution and levels, laboratory safety and disposal practices, toxicology and risk assessment and legal and regulatory aspects.

Sponsors of the symposium are the International Association of Environmental Analytical Chemistry and the International Society of Toxicological and Environmental Chemists, in collaboration with different national and international agencies and groups.

The preliminary organizing committee comprises R. W. Frei (Amsterdam, Holland), O. Hutzinger (Amsterdam, Holland), E. Merian (Therwil, Switzerland), F. Pocchairi (Rome, Italy), G. Reggiani (Basle, Switzerland), B. M. Shepard (Washington, U.S.A.) and A. L. Young (Washington, U.S.A.)

Further information may be obtained from Dr. E. Merian, Im Kirsgarten 22, CH - 4106 Therwil, Switzerland.

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NIH PUBLICATION
No. 82-218

44

TARGET ORGAN TOXICITY :
EYE , EAR AND OTHER SPECIAL SENSES

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ENVIRONMENTAL HEALTH PERSPECTIVES

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service–National Institutes of Health
National Institute of Environmental Health Sciences

VOLUME 44, APRIL 1982

HE 20.3559:

SUBSCRIPTION INFORMATION

Subscription rates are as follows: Single copies $8.00, foreign $10.00, domestic $45.00 per year, foreign $56.25 per year. The yearly subscription is for 6 regular issues. Please send check or money order, in advance to the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

The Secretary of Health and Human Services has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through January 31, 1983.

ENVIRONMENTAL HEALTH PERSPECTIVES is a publication of the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services. Opinions expressed herein, however, are not necessarily endorsed nor shared by any of the above agencies.

DHHS publication No. (NIH) 82–218

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Print Schedule: Volume 45 will present the proceedings from the Conference on Phthalate Esters.

Volume 44, April 1982

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Volume 44, April 1982

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Target Organ Toxicity:
Eye, Ear and Other Special Senses

February 2–3, 1981
Arlington, Virginia

Co-sponsored by the
National Institute of Environmental Health Sciences
and the
Society of Toxicology

pages tightly bound

Environmental Health Perspectives
Vol. 44, pp. 1-8, 1982

The Eye and Visual Nervous System: Anatomy, Physiology and Toxicology

by Connie S. McCaa*

The eyes are at risk to environmental injury by direct exposure to airborne pollutants, to splash injury from chemicals and to exposure via the circulatory system to numerous drugs and bloodborne toxins. In addition, drugs or toxins can destroy vision by damaging the visual nervous system.

This review describes the anatomy and physiology of the eye and visual nervous system and includes a discussion of some of the more common toxins affecting vision in man.

Anatomy of the Eyeball

The eye consists of a retinal-lined fibrovascular sphere which contains the aqueous humor, the lens and the vitreous body as illustrated in Figure 1.

The retina is the essential component of the eye and serves the primary purpose of photoreception. All other structures of the eye are subsidiary and act to focus images on the retina, to regulate the amount of light entering the eye or to provide nutrition, protection or motion. The retina may be considered as an outlying island of the central nervous system, to which it is connected by a tract of nerve fibers, the optic nerve.

As in the case of the brain and the spinal cord, the retina is within two coats of tissue which contribute protection and nourishment. On the outside of the sphere, corresponding to the dura mater, a layer composed of dense fibrous tissue serves as a protective envelope, the fibrous tunic. The posterior part of the fibrous tunic, the sclera, is white and opaque. Although it retains its protective function, the anterior portion, the cornea, is clear and transparent.

Immediately internal to the sclera, and between it and the retina, lies the uvea, a vascular tunic analogous to the pia-arachnoid of the central nervous system. Primarily, the uvea provides nutrients to the eye. The posterior portion of the uvea is the choroid, a tissue composed almost entirely of blood vessels. A second portion of the uvea, the ciliary body, lies just anterior to the choroid and posterior to the corneoscleral margin and provides nutrients by forming intraocular fluid, the aqueous humor. In addition, the ciliary body contains muscles which provide a supporting and focusing mechanism for the lens. The most anterior portion of the uveal tract, the iris, is deflected into the interior of the eye. The iris acts as a diaphragm with a central rounded opening, the pupil, which dilates to allow more light to the retina in dim lighting and constricts in bright lighting. The iris also has some degree of nutritive function, since it acts to help regulate the fluid flow in the eye.

The lens, the focusing mechanism of the eye, is located immediately behind the iris and is supported from the ciliary body by a suspensory ligament, the zonule. The space between the iris and the lens is called the posterior chamber. The anterior chamber consists of the space between the iris and the cornea. Behind the lens is the vitreous, a gel-like, transparent body which occupies the space between the lens and the retina.

The Cornea

The cornea, the window of the eye, is unique because of its transparency. Corneal transparency is dependent on a special arrangement of cells and collagenous fibrils in an acid mucopolysaccharide

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*Division of Ophthalmology, University of Mississippi School of Medicine, Jackson, Mississippi 39216.

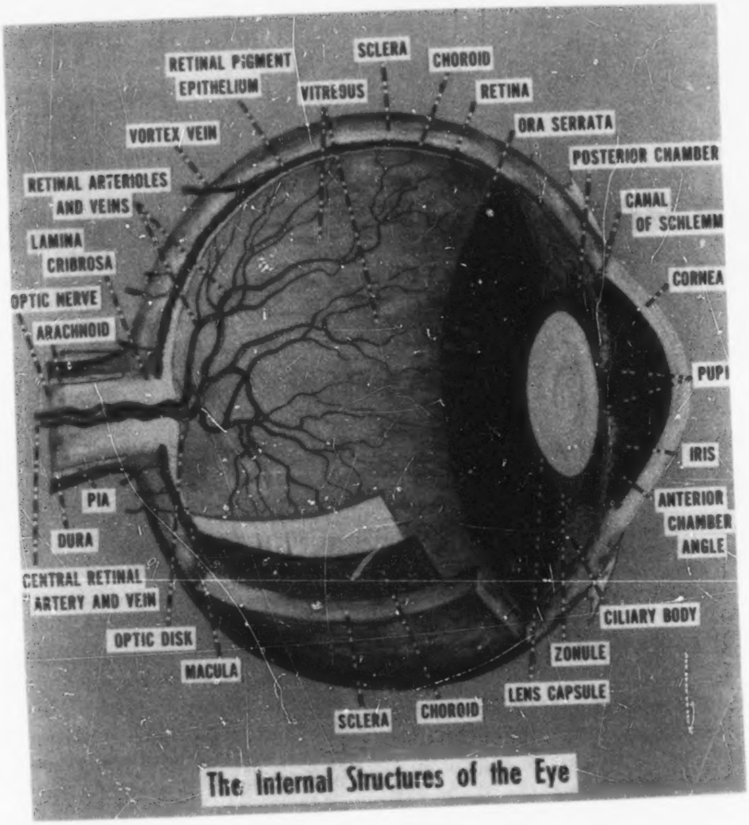
RETINAL PIGMENT EPITHELIUM
SCLERA
CHOROID
VITREOUS
RETINA
VORTEX VEIN
ORA SERRATA
POSTERIOR CHAMBER
RETINAL ARTERIOLES AND VEINS
CANAL OF SCHLEMM
LAMINA CRIBROSA
CORNEA
OPTIC NERVE
ARACHNOID
PUPI
IRIS
PIA
ANTERIOR CHAMBER ANGLE
DURA
CENTRAL RETINAL ARTERY AND VEIN
CILIARY BODY
OPTIC DISK
ZONULE
MACULA
LENS CAPSULE
SCLERA
CHOROID
The Internal Structures of the Eye

FIGURE 1. The internal structure of the eye.

environment, to an absence of blood vessels, and to deturgescence (the state of relative dehydration of corneal tissue). Any toxin interfering with any one of these factors may result in corneal opacification.

Structure of the Cornea

As illustrated in Figure 2, the cornea is composed of five distinct layers: (1) epithelium, (2) Bowman's membrane, (3) stroma, (4) Descemet's membrane and (5) endothelium. In addition, a tear film always covers the cornea of a healthy eye.

The Tear Film. The tear film is made up of three layers. The portion immediately next to the epithelium is rich in glycoprotein produced by the goblet cells of the conjunctival epithelium; a middle, watery layer is secreted by the lacrimal glands; an outside oily layer is produced by the meibomian glands and the glands of Moll and Zeis of the lid. The tear film is essential for the maintenance of the proper optical qualities of the cornea and its deficiency may result in corneal damage.

Corneal Epithelium. The corneal epithelium consists of five or six layers of cells which rest on a basement membrane. It is replaced by growth from its basal cells with perhaps greater rapidity than any other stratified epithelium (*1*).

Bowman's Membrane. Bowman's membrane is not a true basement membrane but is a clear acellular layer which is a modified portion of the superficial stroma. It is a homogenous layer without cells and has no capacity to regenerate if injured.

Corneal Stroma. The stroma makes up approximately 90% of the thickness of the cornea. It consists of alternating lamellae of collagenous tissue parallel to the surface of the cornea. The corneal cells, or keratocytes, are relatively few and lie within the collagen lamellae (*1*).

Descemet's Membrane. Descemet's membrane

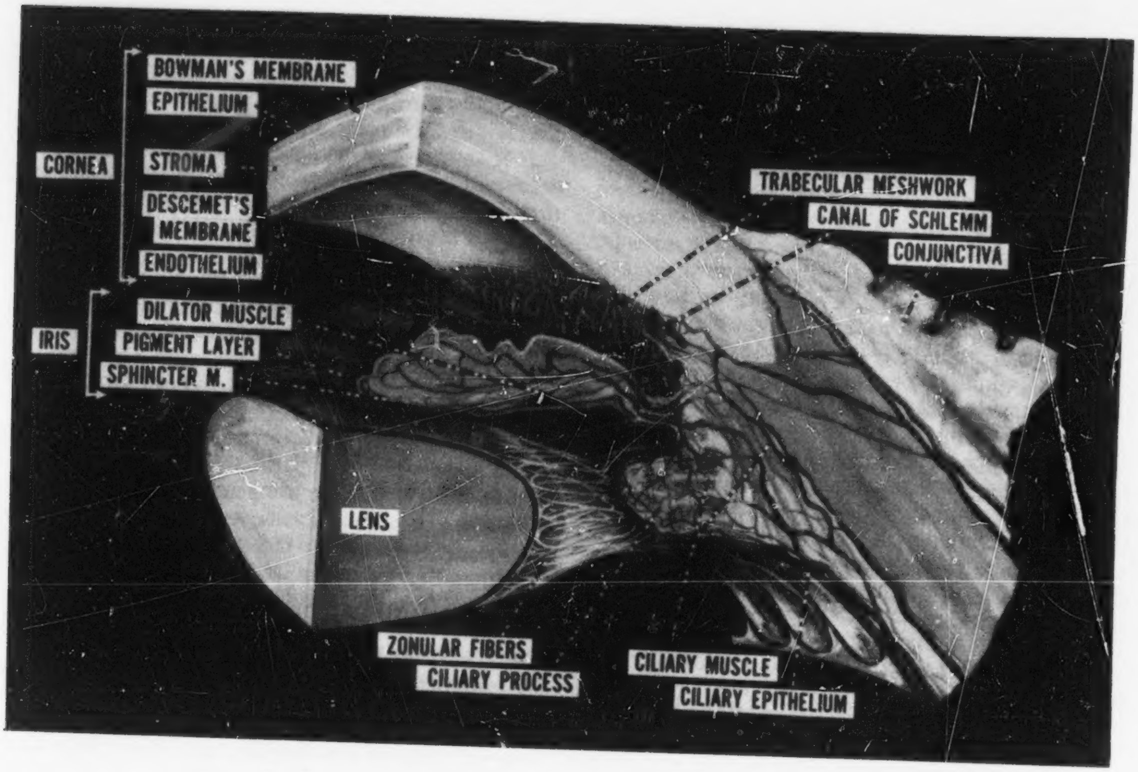
BOWMAN'S MEMBRANE
EPITHELIUM
CORNEA
STROMA
DESCEMET'S MEMBRANE
ENDOTHELIUM
TRABECULAR MESHWORK
CANAL OF SCHLEMM
CONJUNCTIVA
DILATOR MUSCLE
IRIS
PIGMENT LAYER
SPHINCTER M.
LENS
ZONULAR FIBERS
CILIARY PROCESS
CILIARY MUSCLE
CILIARY EPITHELIUM

FIGURE 2. Structure of the anterior compartment of the human eye.

is a strong, homogeneous true basement membrane. It is produced by the endothelial cells and can be regenerated if injured. Descemet's membrane is elastic and is more resistant than the remainder of the cornea to trauma and disease.

Corneal Endothelium. The corneal endothelium consists of a single layer of flattened cuboidal cells. The endothelium does not regenerate and is essential for maintaining dehydration of corneal tissue. Therefore, chemical or physical damage to the endothelium of the cornea is far more serious than epithelial damage. Destruction of the endothelial cells may cause marked swelling of the ornea and result in loss of its transparency.

Permeability of the Cornea o Drugs or Toxins

The penetration of drugs through the cornea is y differential solubility. The epithelium and endohelium will allow the passage of fat-soluble substances, and the stroma will allow the passage of water-soluble substances. All drugs that readily enter the eye after topical application have the ability to exist in equilibrium in solution as ionized and nonionized forms. In the nonionized form they traverse the endothelium and epithelium and in the ionized form penetrate the stroma (*2*).

Toxic Agents Affecting the Cornea

Caustic Agents. Accidental splashing of substances into the eyes is the most common cause of toxic eye injuries. Alkalies and acids may result in rapid, deep, penetrating damage to cornea and deeper eye structures. The injuries produced by alkalies and acids are principally a result of extreme change of the pH within the tissue, an almost immediate action which can be treated only by rapid emergency action. There may be rapid dissolution of epithelium and clouding of corneal stroma from alkalies or coagulation of epithelium by acids.

but many changes appear later, including edema, further opacification, vascularization and degeneration of the cornea. Alkali injury notoriously results in late changes with extreme corneal injury and opacification.

Organic Solvents. Organic solvents, especially those which are good fat solvents, may cause loss of some or all of the corneal epithelium. Yet, even if all the epithelium is lost from the cornea as a result of organic solvents, it generally regenerates in a few days without residual permanent damage (*3*).

Gases, Vapors and Dusts. A great many gases, vapors and dusts induce stinging sensations in the eyes and stimulate tearing. Some of these may cause permanent corneal damage. For example, scarring and distortion of the cornea have disabled workmen many years after chronic industrial exposure to the dusts or vapors resulting from the manufacture of hydroquinone, a chemical used in photographic film development (*3*).

Deposits in the Cornea. Some drugs such as chloroquine, hydroxychloroquine and chlorpromazine produce deposits in the corneal epithelium. These deposits generally appear as fine granules scattered throughout the epithelium. Usually the deposits do not interfere with vision but may defract light and produce an appearance of halos around light. These deposits are generally reversible when the drugs are discontinued.

The Sclera

The sclera is hydrated and has large collagen fibrils arranged haphazardly; therefore, it is opaque and white rather than clear. The sclera has three layers: the episclera, the outer layer; the sclera; and the melanocytic layer, the inner lamina fusca. The episclera, a highly vascular connective tissue, attaches Tenon's capsule to the sclera. The sclera proper is relatively avascular and contains considerable elastic tissue. The sclera is approximately 1 mm thick posteriorly and gradually thins to about 0.3 mm just posterior to the insertions of the recti muscles. Therefore, these sites posterior to the insertion of the muscles are the areas of the eye which are most liable to rupture with trauma to the globe.

The Retina

The sensory retina covers the inner portion of the posterior two-thirds of the wall of the globe. It is a thin structure which in the living state is transparent and of a purplish-red color due to the visual purple of the rods. The retina is a multilayered sheet of neural tissue closely applied to a single layer of pigmented epithelial cells. The sensory retina is attached only at two regions; the anterior extremity is firmly bound to the pigment epithelium at its dentate termination, the ora serrata. Posteriorly, the optic nerve fixes the retina to the wall of the globe. This potential space between the sensory retina and the retinal pigment epithelium may fill with fluid and result in retinal detachment. The fluid usually comes from the vitreous and enters the subretinal space through a tear or hole in the retina (rhegmatogenous or tear-induced retinal detachment). Less commonly, fluid may leak from blood vessels and cause an exudative retinal detachment.

The retina is 0.1 mm thick at the ora serrata and 0.23 mm thick at the posterior pole. It is thinnest at the fovea centralis, the center of the macula. The fovea may suffer irreparable damage in a brief period of separation from its only blood supply, the underlying choriocapillaris, during retinal detachment.

Composition of the Sensory Retina

The sensory retina is composed of highly organized tissue consisting of nine histologic layers resting on pigment epithelium. From the outside of the eye the layers are in the following order: (1) the layer of rods and cones; (2) the external limiting membrane; (3) the outer nuclear layer; (4) the outer plexiform layer; (5) the inner nuclear layer; (6) the inner plexiform layer; (7) the ganglion cell layer; (8) the nerve fiber layer; (9) the internal limiting membrane.

Retinal Pigment Epithelium

The pigment epithelium consists of a single layer of cells which is firmly attached to the basal lamina of the choroid and loosely attached to the rods and cones. Microvilli form the apical parts of the cells and project among the rods and cones. The pigment granules consist of melanoprotein and lipofuscin.

The functions of the pigment epithelium are not completely understood. It produces pigment which acts to absorb light. Also, it has phagocytic functions and provides mechanical support to the processes of the photoreceptors.

Photoreceptor Cells of the Retina

The rods and cones, the light receptive elements of the retina, transform physical energy into nerve impulses. Transformation of light energy depends on alteration of visual pigments contained in the rods or cones.

Rhodopsin, a derivative of vitamin A, is the visual pigment of the rods. Rhodopsin is composed of retinal (vitamin A aldehyde) bound to a large protein, opsin. The retinal is the same in both rods and cones but the protein moiety differs. Light isomerizes the retinal from the 11-*cis* to an all-*trans* shape, releasing the retinal from the opsin. The chemical sequence following the isomerization of retinal produces a transient excitation of the receptor which is propagated along its axon. The bipolar cell transmits this information to the inner plexiform layer where it is modified through connections between amacrine, bipolar, and ganglion cells. The ganglion cells pass this analyzed information to the brain (*4*).

Toxins Affecting the Retina

Chloroquine. A definite association of retinopathy with chloroquine was made in 1959 (*5*), and subsequently more than 200 cases have been reported. There is a definite relationship with retinopathy and daily and total doses. Most cases of chloroquine retinopathy occur at a dosage level of 500 mg per day or more. A total dosage of 100 g can be retinopathic. The risk, however, significantly increases as the total dose becomes greater than 300 g (*6*).

Disturbance of the macula is almost always present in chloroquine retinopathy. Initially it may present as edema followed by development of pigmentary changes. The latter may vary from an extremely subtle abnormality to one having a "bull's eye" appearance to complete loss of macular pigment.

Chloroquine-induced retinal damage occurs primarily at the level of the pigment epithelium and rods and cones. There is loss of the rods and cones with migration of pigment from the adjacent epithelium into the inner retinal layer. Electron microscopic examination has shown additional abnormalities of the ganglion cells consisting of membranous cytoplasmic bodies and clusters of curvilinear tubules (*7*).

The visual prognosis is guarded when a retinopathy secondary to chloroquine develops. In a significant number of cases there is a progressive loss of visual acuity which can continue for several years following the discontinuation of chloroquine (*8*). Hydroxychloroquine causes a retinopathy similar to that of chloroquine (*9*). Visual field testing with a red test object has been recommended as the most reliable indicator of impending retinal toxicity to chloroquine in man (*10*).

Thioridazine. Thioridazine is one of the most commonly used phenothiazines in the treatment of serious psychiatric illness. Its principal ocular toxicity is retinopathy. There is a 75% incidence of retinopathy at a daily dosage level of 1200-2100 mg/day (*11*). A pigmentary mottling of the macula and posterior pole can be observed associated with decreased visual acuity and pericentral and central scotomata (*12*). Improvement in visual acuity is common after the drug is stopped.

Quinine. Quinine may cause ocular damage when taken in large doses. It affects the ganglion cells and nerve fibers resulting in degeneration.

Aqueous Humor

Aqueous humor, contained in the anterior compartment of the eye, is produced by the ciliary body and drained through outflow channels into the extraocular venous system. The aqueous circulation is a vital element in the maintenance of normal intraocular pressure (IOP) and in the supply of nutrients to avascular transparent ocular media, the lens and the cornea. Circulatory disturbance of the aqueous humor leads to abnormal elevation of the IOP, a condition known as glaucoma, which can ultimately lead to blindness.

Aqueous Humor Formation

Formation of aqueous humor is dependent upon the interaction of complex mechanisms within the ciliary body, such as blood flow, transcapillary exchange and transport processes in the ciliary epithelium. Maintenance of the IOP is controlled by a delicate equilibration of aqueous humor formation and outflow; aqueous formation and ocular blood flow are in turn influenced by the IOP (*13*).

Formation of Aqueous by the Ciliary Epithelium

The ciliary epithelium is composed of two layers, the outer pigmented and the inner nonpigmented epithelium. ATPase is responsible for sodium transport to the posterior chamber and for aqueous formation. It is found predominantly in the nonpigmented epithelium (*14*).

Chemical analysis of the aqueous humor indicates that this fluid is not a simple dialysate or ultrafiltrate of the blood plasma (*15*). Continuous aqueous production by the ciliary processes requires an active mechanism demanding metabolic energy. Aqueous humor formation is thereby thought to be due to a secretory mechanism in the ciliary epithelium together with ultrafiltration from the capillaries in the ciliary processes. The secretory mechanism involves active transport of electrolytes, coupled fluid transport, and carbonic anhydrase action.

Aqueous Humor Circulation and Drainage

The anterior ocular compartment containing aqueous humor consists of two chambers of unequal volume, the anterior and posterior chambers (Fig. 2). Communication between the anterior and posterior chambers occurs through the pupil. The aqueous humor is secreted by the ciliary processes into the posterior chamber from which it flows into the anterior chamber. It is drained from the anterior chamber into the extraocular venous systems through porous tissue in the iridocorneal angle and Schlemm's canal (in man and primates) or venous plexus (in lower mammals). This drainage system is called the conventional drainage route. In man and primates, some aqueous leaves the eye by bulk flow via the ciliary body, suprachoroid, and sclera to the episcleral space; this route is called the uveoscleral drainage route or unconventional route.

Effect of Corticosteroids on Intraocular Pressure

Topical application of corticosteroids to normal human eyes occasionally results in IOP elevation accompanied by an increase in the outflow resistance (*16*). After discontinuation of steroids, the IOP usually returns to normal levels but may remain irreversibly elevated. The hypertensive IOP response to steroids appears to be genetically determined (*17*).

The Lens

The lens is a biconvex, transparent, and avascular structure. It is suspended behind the iris by the zonule of Zinn, a suspensory ligament, which connects it with the ciliary body. The lens capsule is a semipermeable membrane which will admit water and electrolytes. A subcapsular epithelium is present anteriorly. Subepithelial lamellar fibers are continuously produced throughout life. The nucleus and cortex of the lens are made up of long concentric lamellae each of which contains a flattened nucleus in the peripheral portion of the lens near the equator.

Function of the Lens

The lens acts to focus light rays upon the retina. To focus light from a near object, the ciliary muscle contracts, pulling the choroid forward and releasing the tension on the zonules. The elastic lens capsule then molds the pliable lens into a more spherical shape with greater refractive power. This process is known as accommodation. With age, the lens becomes harder and the ability to accommodate for near objects is decreased.

Composition of the Lens

The lens consists of about 65% water and about 35% protein (the highest protein content of any tissue of the body). Potassium is more concentrated in the lens than in most body tissues and ascorbic acid and glutathione are both present in the lens. It contains no nerve fibers or blood vessels; therefore, its nutrition is derived from the surrounding fluids. Mechanical injury to the lens or damage from altered nutrient concentration in the aqueous may result in cataract formation.

Cataract

A cataract is a lens opacity. Senile cataract is the most common type and is usually bilateral. Traumatic cataract, congenital cataract, and cataracts secondary to diabetes mellitus, galactosemia, other systemic diseases, and toxins are less common.

Cataract Formation. Cataractous lenses exhibit protein alteration, lens edema, vacuole formation, necrosis, and disruption of the lens fibers. Cataract formation is characterized by a reduction in oxygen uptake and an initial increase in water content followed by dehydration. Sodium and calcium content is increased while potassium, ascorbic acid, protein, and glutathione content is decreased.

Sugar Cataracts. Examples of sugar type cataracts are those produced by galactose, xylose and glucose. Galactose and xylose administered in large amounts to animals and blood glucose in excess, as in severe diabetes with persistent severe hyperglycemia, can produce cataract.

Sugars in high concentration in the aqueous humor readily enter the lens and are converted to sugar alcohols by aldose reductase (galactose to dulcitol or galactitol, glucose to sorbitol, and xylose to xylitol). The sugar alcohols accumulate in the lens and have an osmotic effect causing water to enter the lens and causing lens cells and fibers to swell and become disrupted. These changes lead to formation of cataract (*18*).

Corticosteroid Cataracts. Posterior subcapsular cataracts have been produced by a variety of glucocorticoids used medically for long periods either systemically or applied to the surface of the eye. The toxicologic mechanism of cataract induction by the corticosteroids has not been defined.

Toxic Cataracts. Many cases of toxic cataract appeared in the 1930s as a result of ingestion of dinitrophenol, a drug taken to suppress appetite. Triparanol (MER/29) was also found to cause cataract. Echothiophate iodide, a strong miotic used in the treatment of glaucoma, has also been reported to cause cataract.

The Vitreous

The vitreous is a clear, avascular, gel-like body which comprises two-thirds of the volume and weight of the eye. It fills the space bounded by the lens, retina, and optic disc. Its gelatinous form and consistency is due to a loose syncytium of long-chain collagen molecules capable of binding large quantities of water. The vitreous is about 99% water; collagen and hyaluronic acid make up the remaining 1%.

The Visual Pathway

The visual pathway from the retina may be divided into six levels: (1) the optic nerve, (2) the optic chiasm, (3) the optic tract, (4) the lateral geniculate nucleus, (5) the optic radiation and (6) the visual cortex.

Anatomy of the Optic Nerve

The optic nerve consists of about 1 million axons arising from the ganglion cells of the retina. The nerve fiber layer of the retina is comprised of these axons and they converge to form the optic nerve. The orbital portion of the nerve travels within the muscle cone to enter the bony optic foramen to gain access to the cranial cavity. The optic nerve is made up of visual fibers (80%) and afferent pupillary fibers (20%).

The Optic Chiasm

After a 10 mm intracranial course, the optic nerves from each eye join to form the optic chiasm. At the optic chiasm the nasal fibers, constituting about three-fourths of all the fibers, cross over to run in the optic tract of the opposite side.

The Optic Tract

In the optic tract, crossed nasal fibers and uncrossed temporal fibers from the chiasm are rearranged to correspond with their position in the lateral geniculate body. All of the fibers receiving impulses from the right visual field are projected to the left cerebral hemisphere; those from the left field to the right cerebral hemisphere. Each optic tract sweeps around the hypothalamus and cerebral peduncle to end in the lateral geniculate body with a smaller portion carrying pupillary impulses continuing to the pretectal area and superior colliculi.

The Lateral Geniculate Nucleus

The visual fibers synapse in the lateral geniculate body. The cell bodies of this structure give rise to the geniculocalcarine tract, the final neuron of the visual pathway.

The Optic Radiation

The geniculocalcarine tract passes through the posterior limb of the internal capsule and then fans into the optic radiation which traverses parts of the temporal and parietal lobes en route to the occipital cortex.

The Visual Cortex

Optic radiation fibers representing superior retinal quadrants terminate on the superior lip of the calcarine fissure, and those representing inferior retinal quadrants end in the inferior lip. The macula is represented in a large region posteriorly, and retinal areas close to the macula are represented more anteriorly.

Toxins Affecting the Visual Nervous System

Methanol. Methyl alcohol (wood alcohol) has long been used as an intoxicating drink either accidentally or as an adulterant of ethyl alcohol. Its metabolic product, formaldehyde, can cause marked destruction of the ganglion cells of the retina as well as degeneration of nerve fibers in the optic nerve resulting in permanent blindness.

Nutritional Amblyopia (Tobacco-Alcohol Amblyopia). Nutritional amblyopia is the preferred term for the entity sometimes referred to as tobacco-alcohol amblyopia. Persons with poor dietary habits, particularly if the diet is deficient in thiamine, may develop centrocecal scotomas that are usually of constant density. Bilateral loss of central vision is present in over 50% of patients, reducing visual acuity below 20/200. Reduction in alcohol and tobacco usage plus adequate diet and thiamine is usually effective in curing the disease.

Drugs or Toxins Causing Papilledema. Papilledema as a toxic manifestation of drugs or chemicals can occur as an accompaniment of optic neuritis or as a manifestation of elevation of intracranial pressure. The substances that have most clearly produced papilledema by causing increase in intracranial pressure are the corticosteroids, ethylene glycol, nalidixic acid, tetracycline and vitamin A.

Drugs or Toxins Causing Optic Neuritis. The diagnosis of optic neuritis has usually been made on the basis of clinical observations of reduction of vision associated with central scotomas combined with ophthalmoscopically visible changes in the optic nerve head, particularly hyperemia with variable degrees of edema. Some of these agents are ethambutol, lead, chloramphenicol, isoniazid and digitalis.

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Environmental Health Perspectives
Vol. 44, pp. 9-13, 1982

Structure and Function of the Ear and Auditory Nervous System

by Merle Lawrence*

The transduction from sound vibrations to a nerve ending stimulus takes place in the organ of Corti. This process, resulting from fluid particle movement of a magnitude equivalent to the dimensions of the electron cloud around an atom, involves more than a simple deformation of the sensory cell.

The fluid environment of these cells must be such that it provides sufficient oxygen and nutrients for the cells' survival and at the same time maintains a condition to enhance the biophysical process. The spiral capillaries beneath the tunnel of Corti furnish the metabolites for the Cortilymph surrounding the sensory cells. The capillaries of the stria vascularis provide this structure, an organ in its own right, so that it can maintain the high potassium concentration and positive potential of endolymph which are necessary for the transduction process.

The stria vascularis itself and its blood supply and the sensory cells and their blood supply are all vulnerable to attack by various toxic agents, thus altering the homeostatic condition and resulting in a hearing loss.

Of the two basic peripheral processes of hearing, sound conduction and sound conversion to a coded nerve impulse, certainly the latter is the more complicated and mysterious. It is here that an extremely minute molecular disturbance is analyzed and a stimulus to a diverse network of fine unmyelinated nerve fibers is produced. To maintain a living organ in a condition to accomplish this feat requires a most delicate balance of the chemical environment. The arrangement is such that there is a fluid system within a fluid system (Fig. 1), and the mechanisms of chemical exchange between these two are far from understood.

The Labyrinths

The bony labyrinth within the petrous portion of e temporal bone is filled with perilymph which is nnected with the cerebral spinal fluid (CSF) by e cochlear aqueduct. The patency of this duct aries among the species and even in different ages of life in man. During fetal life and in the ewborn human this aqueduct is wide open, but in the adult it becomes quite narrow and passes through a long bony channel.

At the labyrinthine end, the cochlear aqueduct opens into the scala tympani of the inner ear in the

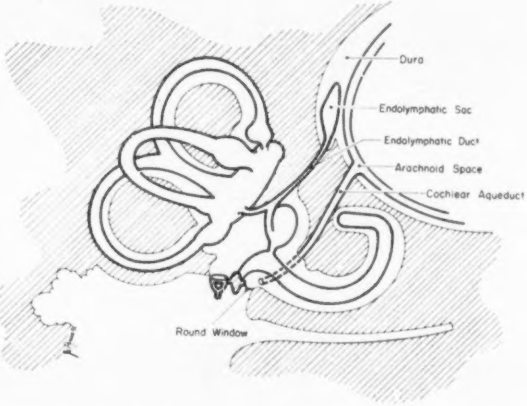

FIGURE 1. The labyrinths of the ear. The bony labyrinth is filled with perilymph which differs slightly from the cerebrospinal fluid to which it is connected. The endolymphatic system surrounded by perilymph is a closed but not stagnant system.

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region of the round window beneath the basilar membrane. As the scala tympani, comprising the space between the basilar membrane and the bony wall of the otic capsule, twists with the spirals of the cochlea, it meets, at the apex through an opening, the helicotrema, with the scala vestibuli. The perilymph continues to fill this scala as it winds back down the turns of the cochlea between the bony separation between turns and Reissner's membrane. The bony labyrinth of the scala vestibuli widens at the base to surround the membranous utricle and saccule and then continues around the semicircular canals. The footplate of the stapes borders this enlarged vestibule of perilymph and conveys its minute motion to the endolymph through the perilymph and Reissner's membrane.

Because the cochlear aqueduct enters the perilymphatic channels at one end of the bony labyrinth it seems unlikely that when everything is normal CSF flows into the inner ear. More than likely, and there is evidence for this, perilymph is produced within the inner ear and is slowly absorbed into the CSF system.

More sodium and protein are added to perilymph somewhere within the perilymphatic system. In the region of the spiral ligament above Reissner's membrane (Fig. 2), there is a capillary network that may well be responsible for providing perilymph with those chemical characteristics that make it different from CSF. The capillaries are close to the surface and are arranged in a way to suggest the formation of a plasma filtrate.

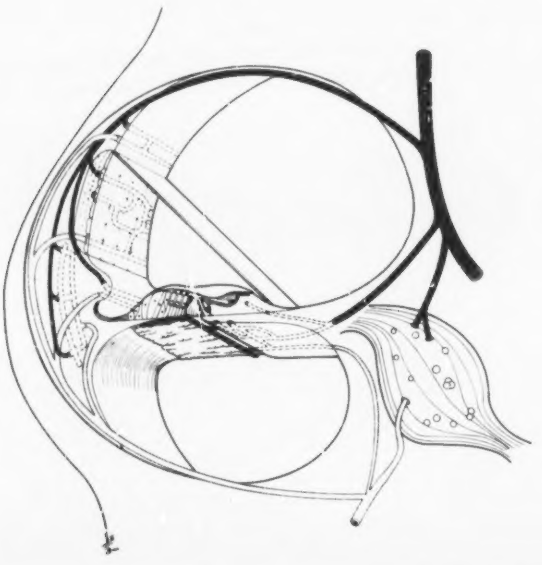


FIGURE 2. The cochlear duct in cross section shows the various capillary areas referred to in the text. The region about which something is known are (a) the capillaries in the spiral ligament above Reissner's membrane, (b) the stria vascularis, (c) the spiral capillaries beneath the tunnel of Corti.

The endolymphatic fluid is a closed system separated from perilymph by Reissner's membrane, basilar membrane, and the membranous walls of the utricle, saccule, and semicircular canals. Continuous with the duct connecting the utricle and saccule midway between the cochlear and vestibular complexes is the endolymphatic duct which, like the perilymphatic aqueduct, passes through bone to end in a closed sac within the folds of the dura. Like perilymph, endolymph is not a stagnant pool. The fluid is generated and its ionic contents controlled within the membranous labyrinth, all the while slowly moving in the direction of the endolymphatic duct to be absorbed in the endolymphatic sac.

The blood supply to the membranous labyrinth is separate from that of the otic capsule. The cochlear artery, entering the internal auditory meatus is a terminal branch of the vertebral-basilar artery complex. After entering the modiolus the artery coils around the nerve, giving off branching arterioles which supply capillaries to the various parts of the labyrinthine structures.

The Stria Vascularis

In a spiral groove (Fig. 2) of the otic capsule around the coils of the cochlea is the spiral ligament. This forms one boundary of the triangular-shaped scala media. Along the surface facing the endolymph is a specialized structure, the stria vascularis, that provides for the peculiar characteristics of the endolymph.

The stria vascularis is a unique structure with specialized cells. These have been described mostly in guinea pig from observations made by both light and electron microscopy. Kimura and Schuknecht (*1*) describe, in humans, the ultrastructure of the three types of cells making up the stria vascularis. The cells on the endolymphatic surface are the marginal or dark cells whose nuclei are close to this surface. On the opposite surface of these cells the membrane is deeply corrugated, interlocking with the cells of the intermediate and basal cells. The luminal surface of the marginal cells shows varying numbers of microvilli and pinocytotic invaginations. An extensive network of tubules filled with a diffuse substance lies close to the luminal surface, and marginal cells adjacent to each other are interlocked with numerous infoldings.

The intermediate or light cells are fewer in number than the marginal cells with which they nterlock. They contain a rather large number of itochondria.

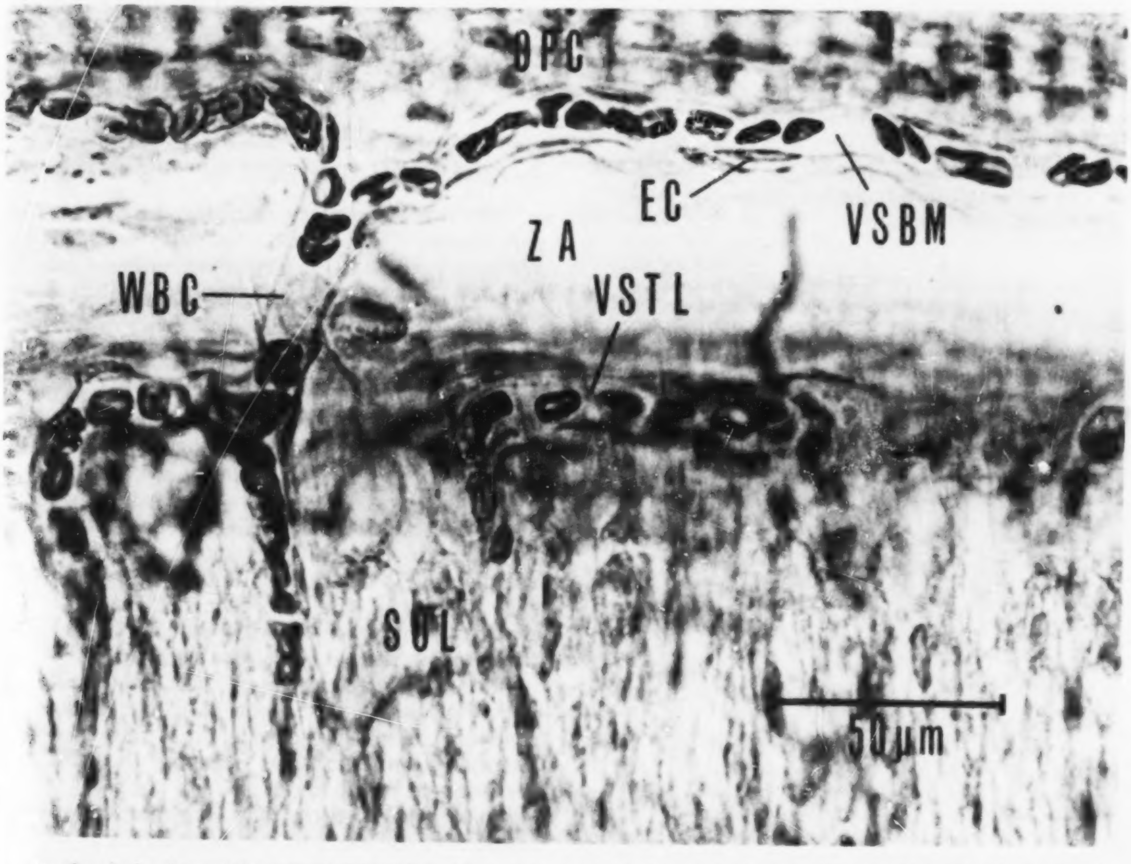
The basal cells lie next to the spiral ligament. hey are flat and long with cell processes that xtend a short distance toward the marginal cells.

Jahnke (*2*), using freeze-fracture techniques in e guinea pig, adds that the marginal cells appear provide a barrier between the endolymphatic ace and the intracellular spaces. But these spaces re very tightly sealed to the spiral ligament so that spaces appear to make up a compartment.

he capillaries of this area enter through the sal cell layer and pass among the intermediate d marginal cells. Smooth muscle cells and neural ements are absent in these capillaries whose dothelial cell walls are extremely thin but not nestrated.

The stria vascularis whose marginal cells lie between the capillaries and endolymph is responsible for maintaining the high potassium content of the endolymph. Along with this high potassium, endolymph has a positive electrical (approximately +80 mV) with respect to perilymph. The source of this potential has been localized to the stria vascularis (*3*) and is believed to be the result of the combined effect of an electrogenic pump and a potassium diffusion potential (*4*): the electrogenic component generating + 100 mV and the potassium diffusion potential accounting for −20 mV. It is further theorized that the pump is located in the membrane of the marginal cells facing the endolymph.

It appears then that the stria vascularis is an organ in its own right. It has a high rate of metabolism and requires oxygen from its own capillaries. The oxygen that can be measured by an electrode in the scala media diffuses out from the stria.



RE 3. A view beneath the tunnel of Corti from the scala tympani side. The region beneath the tunnel is much thinner than the est of the basilar membrane: (ZA) zona arcuata (area beneath the tunnel); (EC) endothelial cell of capillary; (OPC) outer pillar cell; SOL) spiral osseous lamina; (VSBM) vessel of the basilar membrane; (VSTL) vessel of the tympanic lip. (WBC) white blood cell.

The Sensory Cells

The sensory cells of the organ of Corti are protected from endolymph and get their oxygen and nutrients from the spiral vessels.

The capillaries of the osseous spiral lamina emerge beneath the organ of Corti to form two parallel systems lying beneath the feet of the pillar cells, thus bordering the tunnel (Fig. 3). The distance between these capillaries and the hair cells is generally less than 50 μm, while the distance from capillary to tunnel fluid is only a few microns (*5*). The basilar membrane, the organ of Corti, and these capillaries are so situated that fluid exchange is facilitated. The feet of the inner pillar cells rest on the edge of the spiral lamina between the habenula perforata and the attachment of the basilar membrane. From this attachment to a point beneath the outer pillars there is only a thin fibrous layer separating the fluid spaces of the tunnel and the lamella cells on the scala tympani side.

As described, perilymph fills the scala tympani beneath the basilar membrane and easily passes through the thin area of the basilar membrane, named by Corti as the zona arcuata, between the pillars. This Cortilymph (*6*) within the organ of Corti differs from perilymph by the oxygen and nutrients that diffuse from the spiral capillaries.

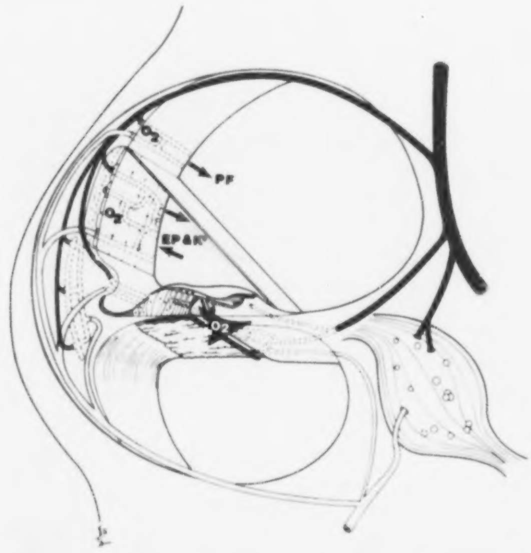


FIGURE 4. Functions of capillary areas vulnerable to attack by toxins: (PF) plasma filtrate providing perilymph with its chemical constituents; (EP and K^+) endolymphatic potential and high potassium controlled by the stria vascularis; (O_2) the oxygen sources for the areas maintaining microhomeostatis.

Several lines of evidence have indicated the role of the spiral vessels in the maintenance of Cortilymph. Vosteen (*7*) reviewed a number of experiments that indicate the spiral vessels as the main source of oxygen for the sensory cells. In other studies, direct measures of oxygen availability were made simultaneously (*8*) in the tunnel fluid and endolymph as the animal was made anoxic by shut-off of respiratory air. Oxygen concentration in the tunnel changed rapidly with the anoxic condition of the animal whereas the oxygen concentration of the endolymph responded much more slowly. Apparently the spiral vessels provide the oxygen directly to the sensory cells.

Adequate function of these sensory cells, the *sine qua non* of hearing, requires more than a sufficient oxygen supply. Even though the sensory cells are protected from endolymph, the positive endolymphatic potential plays an important role. So the capillary blood supply to both the tunnel of Corti and the stria vascularis is most important (Fig. 4). However, the oxygen released by the stria vascularis capillaries is utilized by the stria vascularis itself (*9*, *10*) and is not a supply for the sensory cells.

Areas of Vulnerability

There are then several capillary areas and transport functions that can be affected by toxic agents. We have reported (*11*) an absence of blood cells in both the stria vascularis and in the spiral vessels following an acoustic overstimulation in the guinea pig. Hawkins (*12*) has reported that for adequate dosages of both quinine (quinine dihydrochloride) and salicylates (sodium salicylate) there results a partial occlusion of the spiral capillaries by the swollen endothelial cells which block the passage of blood cells.

Pike and Bosher (*13*) have shown that intravenous furosemide in guinea pigs produces ultrastructural abnormalities in the marginal cells of the stria vascularis with shrinkage of intermediate cells and enlargement of the intercellular spaces. These changes are correlated with changes in the ion-transporting mechanism of the stria (*14*).

Ototoxic drugs may also affect the capillaries above Reissner's membrane (*15*), and changes in the ionic content of the endolymph may affect the tectorial membrane, thus changing the adequate performance of the organ of Corti itself (*16*).

It is apparent, then, that both morphology and function of the ear and its physiological processes are subject to changes resulting in alterations of the microhomeostatic conditions of the organ of Corti. Some drugs, such as the salicylates, will produce a rtial hearing loss that may exist as long as the ig is taken but when stopped, hearing returns to rmal. Of the physiological processes just described might ask which ones are able to function at lf normal." Perhaps all of them, but most oxic effects from the antibiotics and diuretics n to be permanent. However, the endolym- tic potential upon which adequate function of rgan of Corti seems to depend can be reduced maintained at lowered levels by reduction in l oxygen.

xperiments (*17*) have shown that the reduced ocochlear potential from partial anoxia can be ntained over relatively long periods of time. haps such an effect can account for the revers- hearing loss seen in some deafnesses resulting m toxins.

This brief outline of the morphology and possible physiological functions of capillary areas and cell groups covers only in a very sketchy way the processes that we know about. Why certain toxins have an affinity for the ear may be related to the lack of replaceable cell groups and the poor redundancy of the blood supply.

The support of PHS Grants NS-05785 and NS-11731 is acknowledged.

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Environmental Health Perspectives
Vol. 44, pp. 15-21, 1982

Olfaction: Anatomy, Physiology nd Behavior

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The anatomy, physiology and function of the olfactory system are reviewed, as are the normal effects of olfactory stimulation. It is speculated that olfaction may have important but unobtrusive effects on human behavior.

The sense of smell has been neglected in comparison to other senses. Among the reasons for this are (a) inaccessibility of anatomical structures, (b) limited theoretical knowledge about the nature of the physical stimulus, (c) difficulty with generating and presenting stimuli and (d) the belief that the olfactory sense is not important to man. Some of these reasons remain problems, but techniques for dealing with them are being devised (*1*). No recent major advance in olfactory stimulus theory has occurred. The belief about the unimportance of olfaction has been questioned recently and is discussed in this paper.

The major reason for reviewing olfaction in a toxicological/environmental context is that this neural tissue is directly exposed to air pollutants. Since these olfactory receptors appear to function by retaining molecules on their surface, airborne pollutants and toxic substances have a high potential producing olfactory damage. The olfactory sys- n is perhaps the most vulnerable neural tissue in ms of airborne pollutants, since the receptors directly exposed and are also exposed via the latory system to bloodborne toxicants.

his review describes the anatomy and physiol- of the olfactory system and also discusses the avioral implications of this sense. In the limited space available only overall conclusions can be given. Key references will be cited for further study.

Olfactory Anatomy

Peripheral Anatomy and Physiology

Nasal Passages. Peripheral features of the olfactory system are illustrated in Figures 1 and 2. Due to shape of the turbinate bones, only about 2% of inspired air reaches the olfactory epithelium during normal respiration (*4*). During passage over the nasal epithelium, temperature and humidity of inspired air are altered to body temperature and nearly saturation (*5*). Particulate matter in inspired air is deposited on nasal mucosa and swept toward the pharynx by the cilia. Mucous is supplied to the nasal epithelium by glands and goblet cells within the mucosa.

There is a cyclic variation in the flow resistance of each nasal passage (*6, 7*) which is due to the regular constriction and dilation of the mucosal venous cavernous tissue (*8*) and is thought to hypothalamically regulated (*9*) via the Vidian nerve (*10*). At the peak of the cycle, one nasal cavity has low flow resistance and the mucosa is moist while the other has high flow resistance and dry nasal mucosa (*11*).

Olfactory Epithelium. The human olfactory epithelium, located at the apex of the nasal cavities (Figs. 1 and 2), encompasses about 2-4 cm^2 and contains about 10×10^6 receptor cells (*12*). It is covered by a mucous layer secreted by Bowman's gland.

The olfactory epithelium (Fig. 3) is composed of

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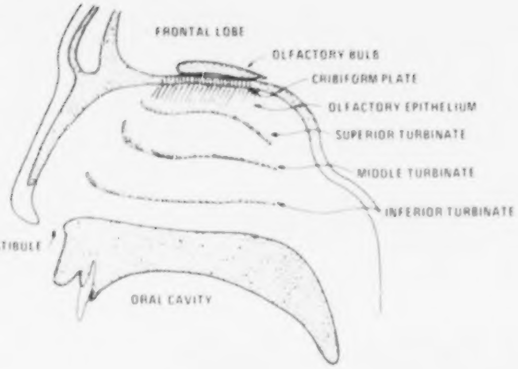
FRONTAL LOBE
OLFACTORY BULB
CRIBIFORM PLATE
OLFACTORY EPITHELIUM
SUPERIOR TURBINATE
MIDDLE TURBINATE
INFERIOR TURBINATE
VESTIBULE
ORAL CAVITY

FIGURE 1. Midsagittal view of nasal cavity, showing location of olfactory structures and turbinates. Adapted from Proetz (*2*).

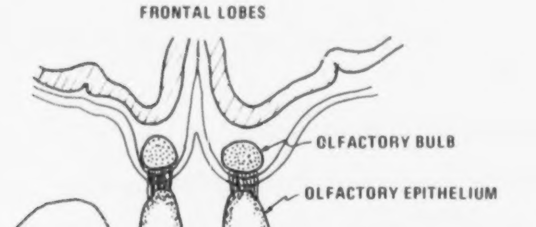
FRONTAL LOBES
OLFACTORY BULB
OLFACTORY EPITHELIUM
SUPERIOR TURBINATE
SEPTUM
MIDDLE TURBINATE

FIGURE 2. Frontal section through nasal cavity showing the location of olfactory structures with respect to the brain. The convoluted shape of the turbinate bones is also shown. Adapted from Schneider (*3*).

receptor cells, basal cells and sustentacular cells. The receptors are bipolar sensory cells and are often compared morphologically and functionally to the bipolar retinal cells. They are capped by immotile cilia that extend about 160μm into the mucous. It is presumed that the receptor sites are located in the cilia (*13*). Unmyelinated axons of receptors extend through the cribriform plate (a perforated area of the ethmoid bone) and synapse within the olfactory bulb (OB). There is a loose topographic mapping from the olfactory epithelium to the olfactory bulb.

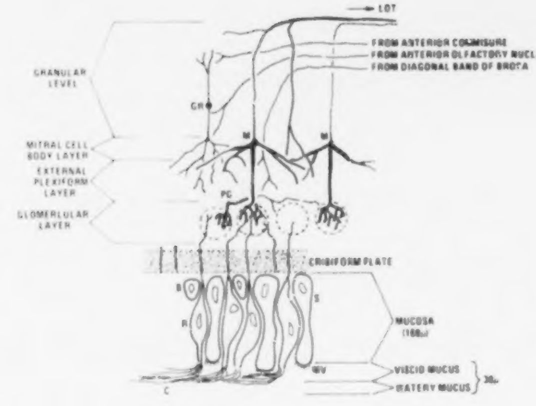
LOT
FROM ANTERIOR COMMISURE
FROM ANTERIOR OLFACTORY NUCLEUS
FROM DIAGONAL BAND OF BROCA
GRANULAR LEVEL
GR
MITRAL CELL BODY LAYER
EXTERNAL PLEXIFORM LAYER
GLOMERULAR LAYER
M
M
PG
CRIBIFORM PLATE
B
S
R
MUCOSA (100μ)
MV
VISCID MUCUS
WATERY MUCUS
30μ
C

FIGURE 3. Cells and connections of the olfactory epithelium an olfactory bulb: (B) basal cell; (C) olfactory cilia; (GR) granula cell; (M) mitral cell; (PG) periglomerular cell; (S) sustentacular cell; (mc) microvilli.

The sustentacular cells, as the name implies, support the receptor cells and provide a secretion with an unknown role and composition (*12*, *14*, *15*). Also located in the olfactory epithelium are basal cells whose function is not known.

Trigeminal Innervation. The non-olfactory nasal cavity is innervated by free nerve endings of the ethmoid branch of the trigeminal nerve (*16*). These endings are found in the epithelium of the pharynx as well. The trigeminal nerve synapses in the trigeminal nucleus in the pons.

Central Olfactory Structures

The connections and ultrastructure of the olfactory sense in the central nervous system (CNS) have been recently reviewed by Shepherd (*17*, *18*). Other reviews (*19-21*) provide background information.

Olfactory Bulb. Major connections of the olfactory bulb (OB) are shown in Figure 3. Axons of receptor cells synapse in glomeruli (spherical areas of dense neuropil) on mitral and periglomerular cells. About 25,000 receptor axons synapse in each glomerulus with 25 mitral cells (a 1,000:1 convergence ratio). Axons of mitral cells form the lateral olfactory tract (LOT) and carry impulses toward

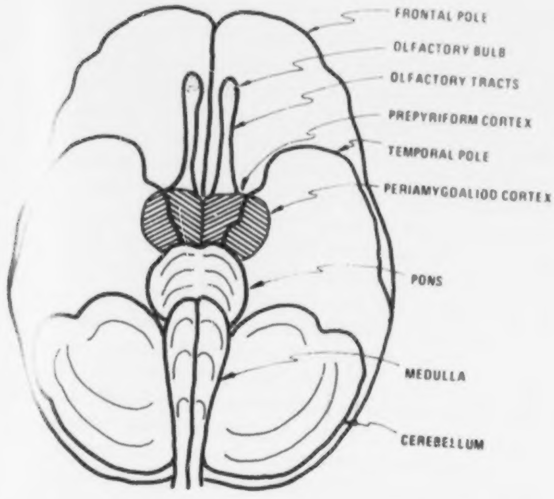
FRONTAL POLE
OLFACTORY BULB
OLFACTORY TRACTS
PREPYRIFORM CORTEX
TEMPORAL POLE
PERIAMYGDALIOD CORTEX
PONS
MEDULLA
CEREBELLUM

FIGURE 4. Ventral view of the human brain. Cross-hatched areas show approximate area of termination of primary olfactory fibers. Structures to which synapses are made underlie these areas.

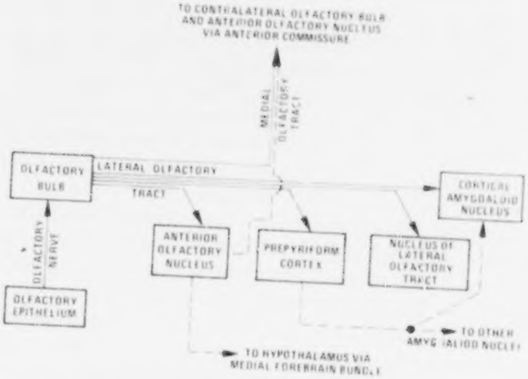
TO CONTRALATERAL OLFACTORY BULB AND ANTERIOR OLFACTORY NUCLEUS VIA ANTERIOR COMMISSURE
MEDIAL OLFACTORY TRACT
OLFACTORY BULB
LATERAL OLFACTORY TRACT
CORTICAL AMYGDALOID NUCLEUS
OLFACTORY NERVE
ANTERIOR OLFACTORY NUCLEUS
PREPYRIFORM CORTEX
NUCLEUS OF LATERAL OLFACTORY TRACT
OLFACTORY EPITHELIUM
TO OTHER AMYGDALIOD NUCLEI
TO HYPOTHALAMUS VIA MEDIAL FOREBRAIN BUNDLE

FIGURE 5. Schematic of principal centripetal connections of the olfactory bulb.

the CNS. Axon collaterals of mitral cells also make connection to other cells within the bulb. Periglomerular cells, which outnumber mitral cells about 20:1, make horizontal connections between glomeruli.

Granular cells, in deeper layers of the olfactory bulb, make dendrodendritic connections with mitral cells and with each other as well as axodendritic connections with centrifugal neurons. Centrifugal neurons synapse mainly with the granule cells and originate in the contralateral olfactory bulb (crossing via the anterior commisure), in the ipsilateral anterior olfactory nucleus and in the ipsilateral diagonal band of Broca. The latter centrifugal fibers extend to synapse with periglomerular cells.

lfactory Cortex. A simplified ventral view of uman brain in Figure 4 shows the approximate of olfactory fiber termination. Principal cen- al cortical connections are shown schemati- n Figure 5. The lateral olfactory tract courses ally over the prepyriform cortex toward the daloid body and along its course fibers branch it and spread across the cortical ventral sur- to synapse in the anterior olfactory nucleus, prepyriform cortex, the nucleus of the lateral tory tract and the cortical amygdaloid nucleus. al cell axons from the lateral olfactory tract axodendritic synapses with pyramidal cells in uter molecular layer of the cortex but do not h deeper layers.

lfactory cortex structures send secondary fibers ich have made synaptic connections with mitral cell axons) to other CNS sites. The anterior olfactory nucleus contributes fibers to the medial forebrain bundle which terminates in the hypothalamus as well as sending centrifugal fibers to the granule cells of the ipsilateral olfactory bulb. Prepyriform cortical fibers are traceable to the amygdala, the hypothalamus and possibly the hippocampus. Amygdala cells fibers send send axons to the hypothalamus, the prepyriform cortex and the hippocampus. This is, of course, not an exhaustive list of secondary fiber connections. Only major connections are noted.

Other multisynaptic CNS olfactory connections have recently been reported (*22-24*). These involve pathways originating in the amygdala and prepyriform cortex, passing through the thalamus and terminating in the orbitofrontal cortex. This represents a neocortical site as opposed to the more classical allocortical (old cortex) and limbic projections.

Physiology and Function

Psychophysiology

An olfactory stimulus consists of airborne chemical molecules within the molecular weight range of approximately 15 to 300. The intensity of the stimulus is a function of the number of molecules of odorous substance in contact with the olfactory epithelium. The rate of perceived increase in intensity with increased odorant concentration is not constant across different odorants but is a log function of concentration with the slope being influenced by water solubility of the odorant (*25*) and chemical functional groups (*26*).

Threshold, usually defined as the stimulus concentration the subject detects 50% of the time, is somewhat more difficult to study. The problem of adaptation (reduced sensitivity) to the stimulus can occur with multiple presentations of the same stimulus. Cross-adaptation may also occur with successive presentations of different stimuli. Measurement of thresholds presents the additional problems of individual variability and technique (*27*), with practice (*28*) and with instrumentation (*29*).

The classification of odor quality has been a puzzle for centuries (*30*). Numerous theories have been created to explain olfactory quality (*31*). Amoore (*32*) postulated and Beets (*33*) elaborated upon a stereochemical theory of olfaction based upon receptor sites on the cilia. These hypothesized sites had different shapes to receive complimentarily shaped primary odor molecules. Their hypothesized primary odors were deduced from organic chemistry literature and were analogous to primary colors in vision. Unfortunately, evidence for such receptor sites and primary odors is poor; correlations with actual perceived odor qualities are far from perfect, and there are notable exceptions to their rules.

Davies (*34*) proposed a puncture and penetration theory of olfaction whereby the odorant molecules actually enter the receptor cell and precipitate depolarization. Odor quality was hypothesized to be determined partly by the rate of diffusion through the membrane and the resistance of the individual receptor membranes to puncturing. There is no evidence that odorant molecules actually penetrate a receptor or that they have different diffusion rates.

Wright (*35*) proposed a molecular vibration theory of odor quality. In this theory, the vibrational frequency of molecules in the far-infrared determines the quality, while volatility, adsorbability and water–lipid solubility determine the potency of the odor. This idea has been widely criticized (*31*, *36*, *37*).

A spatio-temporal model of olfaction has been proposed (*38*). In this chromatographic analogy, the pattern of spread of odorant molecules across the olfactory epithelium determines olfactory quality. There is little solid evidence for support of this new theory.

In summary, while there are many theories about olfaction, most seem not to provide hard evidence in their favor. While most involve the structure of the molecule, it is not clear what physicochemical attribute of molecules makes them odiferous. The unknown nature of the receptor mechanism further complicates the problem.

CNS Olfactory System

Olfactory Bulb. The anatomy of the olfactory bulb suggests that this structure is more than a simple "telephone repeater" station. At the glomerular level, sensory cells fire into a synaptic neuropil of mitral and periglomerular cell dendrites. Since mitral cells are the principal neurons in the olfactory bulb, their apparent function is transmission of sensory data. Even at the glomerular level however, there is considerable data processing because of the network of periglomerular cells. There appears to be odor specificity at the glomerular level (*39*, *40*), and, because of inhibitory synaptic processes, there is considerable spatial and temporal sharpening of input data (*41*, *42*).

As seen in Figure 3, connections in the inner three layers of the olfactory bulb deal with interactions of sensory input data and output from the CNS. These interactions occur in synapses between mitral and granule cells with axons from centrifugal cells. Periglomerular cells also receive centrifugal influence. It has been demonstrated that the synaptic connections in the inner layers of the olfactory bulb form recurrent inhibitory loops (*43*-*45*) which are responsible for the generation of the oscillatory electroencephalogram (EEG) which can be measured with macroelectrodes in the olfactory bulb (*46*). The inner layers of the olfactory bulb, therefore, seem to integrate sensory stimuli with centrifugal impulses and thus perform higher level data processing to aid in olfactory perception and control of olfactory-guided behavior.

Odor Code. The question of how odor information is coded and transmitted to the brain is largely unanswered. There are several lines of evidence, but the basic problems with all of them are that salient stimulus dimensions are not understood and, perhaps as a consequence of these problems, correlations of electrophysiological responses with stimulus properties are rather low, even though statistically significant.

There is mounting evidence for an odor specificity in the mitral cells of the olfactory bulb (*39*, *40*, *47*) which might be ascribable to spatially organized projections from the olfactory epithelium (*48*-*50*). Some evidence for olfactory coding in olfactory bulb EEG has also been reported (*51*). In both the EEG and single unit response data, however, it has been shown that the putative codes also are influenced by habituation and learning (*52*-*55*).

Variations in the meaning of odorants to the organism and the effects of arousal states of the organism can easily be confused for odor codes unless these variables are controlled. Similarly, unless intensity of stimulation is controlled for various odors, the code for intensity might be mistaken for an odor code. While the cited research provides suggestions for further work, there were many uncontrolled variables in these studies and results tained high residual variance. At this time no groundwork has been laid to aid theorists in ntangling pure sensory information from pro- ed and interpreted data. Perhaps the CNS tiplexes these data and generates signals in ch interpretational modifiers are appended to ory data "words."

rain Olfactory Mechanisms. The structures e brain which receive olfactory input data are known to be involved in the regulation of basic viors which are well reviewed by Thompson . Laboratory animals will work to receive elec- l stimulation to the medial forebrain bundle. T hypothalamus is intimately involved in the ulation of hunger, thirst, sexual activity and ep. Lesions in the amygdala and surrounding uctures produce alterations in sexual and social haviors. All of these areas receive impulses from the olfactory bulb, and, indeed, the electrical activity of these centers is sometimes almost completely dominated by such inputs. It is not clear how much of the information transmitted to these CNS centers is sensory data and how much of it has been processed into signals for action. Whatever the nature of the signals which are sent to the CNS, the signals are sent to widespread and important sites. There are recent data indicating that the human amygdala EEG correlates somewhat with odor qualities (*57*).

Behavior

For neurotoxicologists, the importance of anat- ny and physiology rests upon the consequences of sturbances in the CNS on behavior. Behavior is final common pathway. In nonhuman species it vell known that olfactory stimulation can very ngly influence sexual behavior (*58*, *59*) as well social behaviors between and within species, as aggression, territorial defense and identi- ion (*60*, *61*). It is usually assumed, however, olfactory stimuli play only minor roles in ncing the behavior of humans. This assump- is based largely upon introspection about the es of behavior rather than empirical evidence. he case where sensory information is distri- d to limbic system centers, however, it is ques- able whether introspection would yield any infor- ion. Such subcortical input might in fact exert alled "unconscious" influence. If this is the case, uences of odor on human behavior could be quite ortant, especially because of their unobtrusive ure. With all of the attempts in our society at trol of the olfactory environment, by means, ., of perfumes and deodorants, and with all of odiferous environmental pollution, it seems especially important to understand the effects of odors upon humans.

Evidence is beginning to emerge on the role of the olfactory sense on human behavior. Humans can use odors to identify individuals (*62*, *63*) although it is unknown to what extent they normally do so. Humans generate pheromonelike compounds (*64*, *65*), and such pheromones affect sexual attractiveness in both males and females (*66*, *67*). Other social behaviors, in addition to sexual attraction, might well be affected by odor cues (*68*). There are correlations between olfactory acuity in women and menstrual variations (*69*, *70*). There is also evidence that the smell of jasmine flowers inhibits lactation in human females (*71*).

While the findings regarding olfactory effects on human behavior are only suggestive, it is certainly logical on anatomical and physiological grounds that such effects should exist since olfactory connections are made to widespread and important CNS sites (*72*). To assume that the effects are not important might be to overlook a strong and yet unobtrusive effect of the olfactory environment on everyday human behavior. More research on this issue is required.

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Environmental Health Perspectives
Vol. 44, pp. 23-30, 1982

Structure and Function of the Somatosensory System: A Neurotoxicological Perspective

by Joseph C. Arezzo*, Herbert H. Schaumburg* and Peter S. Spencer*

The somatosensory system comprises those elements of the peripheral nervous system (PNS) and the central nervous system (CNS) subserving the modalities of touch, vibration, temperature, pain and kinesthesia. Specific modalities can be associated with unique peripheral receptors, peripheral axons of stereotyped diameter and specific central projection pathways. Several features of the somatosensory system render regions of it vulnerable to a wide variety of toxicants. The present report highlights these features and, furthermore, suggests that analysis of these regions is invaluable in studying the three most common varieties of toxic neuropathy: toxic distal axonopathy, toxic myelinopathy and toxic sensory neuronopathy.

Receptors

Structure and Function

Since the early nineteenth century, various specialized receptors have been associated with the somatosensory system. These receptors, located in skin, hair follicles, joints and muscles, transduce mechanical or thermal energy. Minimally, a receptor includes a peripheral axon terminal of one primary afferent neuron, whose cell body is sited proximally in the dorsal root ganglion. Receptors often include nonneural elements which incorporate and interact with the axon terminal in initiating generator potentials. Multiple generator potentials may summate to the threshold necessary for triggering a nerve impulse (action potential). Three major categories of somatosensory receptors can be identified: mechanoreceptors, thermoreceptors and nociceptors. These categories are further subdivided on the basis of location and rate of adaptation.

Mechanoreceptors are sensitive to nondamaging mechanical disturbances of skin or hair. An example of a slowly adapting position detector is a Type I Iggo corpuscle, featured by a myelinated axon terminating at the base of a small dome-like elevation in the skin (Merkel cells). Displacement of the dome by as little as 5 μm can result in a supra-threshold generator potential within the Merkel cell-axon terminal complex. Mechanoreceptors sensitive to rapid transients are exemplified by the Pacinian corpuscles. The Pacinian corpuscle is principally composed of an outer capsule formed of connective tissue lamellae separated by fluid-filled interlamellar spaces, and an inner core of tightly spaced lamellae (Fig. 1). Proximally, the outer core is penetrated by a canal which contains a single myelinated preterminal axon (7-11 μm across), continuous with a long naked axon terminal deep in the center of the corpuscle. The long terminal axon is elliptical in section, is provided with juxtaposed filopod processes, and has a bulbous ultraterminal ending from whose entire surface filopod processes emanate in a hydra-like array. Filopod processes appear to con-

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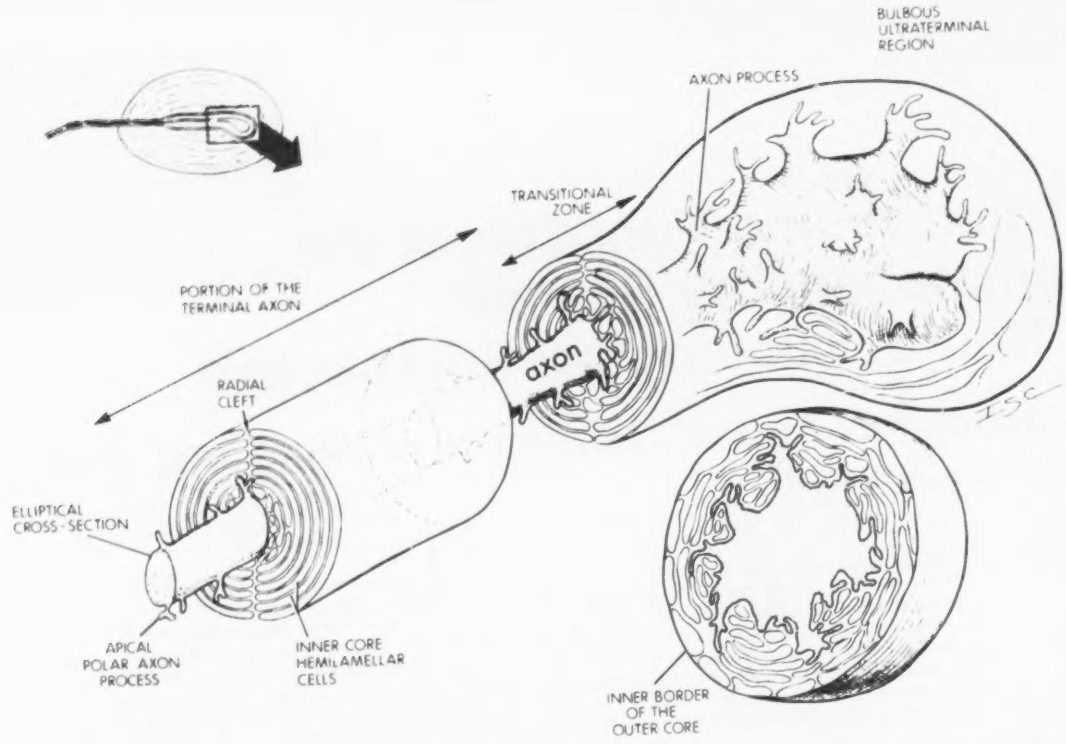

BULBOUS ULTRATERMINAL REGION
AXON PROCESS
TRANSITIONAL ZONE
PORTION OF THE TERMINAL AXON
axon
RADIAL CLEFT
ELLIPTICAL CROSS-SECTION
APICAL POLAR AXON PROCESS
INNER CORE HEMILAMELLAR CELLS
INNER BORDER OF THE OUTER CORE

FIGURE 1. Three-dimensional diagram of a Pacinian corpuscle indicated by the boxed area in the upper left diagram, illustrating the distal end of the terminal segment, the transitional zone and the ultraterminal region. Lower right: a cross-sectional view of the ultraterminal chamber. From Spencer and Schaumburg (*1*).

tact the inner core lamellae. This physical relationship may provide the substrate for transmitting fast pressure changes characteristic of these rapidly adapting vibration receptors (*1*).

Cutaneous thermoreceptors display three consistent properties: static discharge at constant temperature; dynamic response to temperature change; and relative insensitivity to mechanical stimuli. Although both warm and cold skin spots have been repeatedly mapped, no specific encapsulated or corpuscular receptor has been consistently identified. Thermoreceptors most likely are branching matrices of naked axons terminating in the stratum papillare. Their terminations contain numerous small vesicles and mitochondria. Nociceptors for pain also appear to be "free nerve" endings in skin and muscle.

Neurotoxicology

Toxic neuropathy characterized by degeneration in the distal segments of large-diameter axons (distal axonopathy) is a common morphological reaction of the peripheral nervous system to exogenous toxins (*2*, *3*). The neural elements of somatosensory receptors in the hands and feet represent the distal extreme of long afferent fibers, and thus, are particularly vulnerable in the distal axonopathies. Selective receptor inactivation is an early change following exposure to certain neurotoxins, e.g., failure of the generator potential, followed by loss of the axon filopod processes in Pacinian corpuscles, are early physiological and morphological alterations following acrylamide intoxication (*4*, *5*). Toxic sensory neuronopathy, characterized by degeneration of dorsal root ganglion cells, produces degeneration in sensory axons of peripheral nerves and, presumably, of their axon terminals in receptors (*6*).

Peripheral Nerve

Structure and Function

Large nerves, such as the tibial, consist of several fascicles containing numerous myelinated and

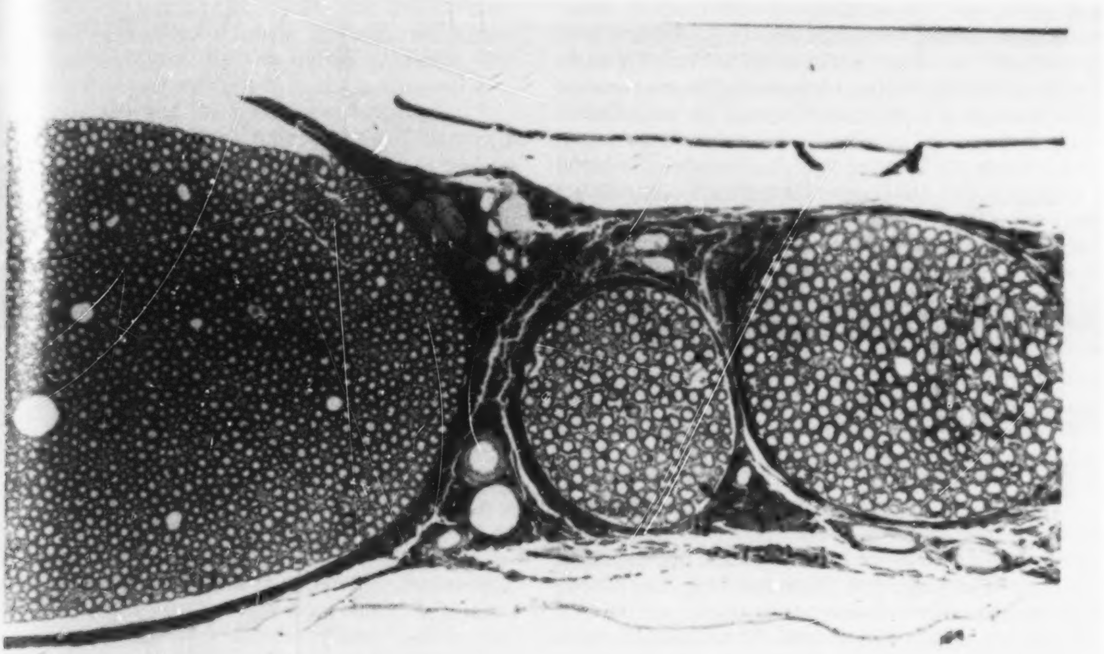


FIGURE 2. Light micrograph of a transverse section of a portion of mammalian tibial nerve and its branches, each containing numerous myelinated axons. Epoxy section, 1 mμ, stained with toluidine blue. × 180.

unmyelinated axons (Fig. 2). Each fascicle is enclosed by a perineurial ensheathment, which serves as a diffusion barrier and accounts for much of the tensile strength of the nerve. Distally, the perineurium fuses with the connective tissue of encapsulated sensory receptors, but does not cover free nerve endings. Proximally, the perineurium surrounds the dorsal root ganglia, which contains the cell body of the primary afferent fiber, and is continuous with the root sheaths. The axon of nerve fibers is ensheathed by a chain of Schwann cells. In myelinated fibers, this ensheathment takes the form of a multilayered membranous structure (myelin), a product of the Schwann cell. Interruptions between each length of myelin sheath, called nodes of Ranvier, occur at regularly spaced intervals along the length of the nerve fiber.

Afferent fibers of the somatosensory system vary in cross-sectional diameter from approximately 0.4 μm to 20 μm. The rate of impulse conduction varies directly with the diameter of the axon. Heavily myelinated axons are fast conductors, while thinly myelinated and unmyelinated axons conduct less rapidly. Subpeaks of the compound action potential can be used to classify axons as to size and conduction velocity. Mechanoreceptors are predominately subserved by medium- and large-size myelinated fibers, while thermoreceptors and nociceptors rely on relatively small myelinated and unmyelinated fibers. However, there is considerable overlap, and no conduction velocity uniquely specifies a receptor class.

Neurotoxicology

The bipolar dorsal root ganglion cell (DRG) and its long, heavily myelinated axons are exquisitely vulnerable to each of the three common types of neurotoxic injury. Distal axonopathies (e.g., acrylamide, *n*-hexane) are characterized by selective degeneration of long large-diameter axons. Therefore, bipolar DRG neurons whose peripheral processes extend the length of the sciatic nerve, and whose central processes traverse the length of the spinal cord to the gracile nucleus, are early affected. Toxic myelinopathies (e.g., hexachlorophene, acetyl ethyl tetramethyltetralin) are associated with bubbling of PNS and CNS myelin sheaths. The long, heavily myelinated axons of the bipolar DRG neuron, extending for great lengths both to CNS and PNS, are consistently involved (7, 8). Substances producing neuronopathies (e.g., doxorubicin) presumably act directly on the soma of neurons (9). Such substances do not have access to most nerve cells because the tight junctions of the endothelial cells of nervous system blood vessels (blood-brain and blood-nerve barrier) prevent leakage. The blood vessels of the DRG are fenestrated, permitting such substances to leak out and selectively damage these neurons (toxic sensory neuronopathy). This produces rapid degeneration throughout the length of peripheral sensory axons and dorsal columns. Sensory nerves in upper and lower extremities are equally affected.

Spinal Pathways

Structure and Function

The spinal cord contains a number of well-defined, modality-specific fiber tracts which ascend from the segmental levels to the brainstem and diencephalon (Fig. 3). The largest of these tracts is the dorsal-column system which forms the dorsal and medial boundaries of the spinal cord. The central projections of dorsal root ganglia course in the medial division of the dorsal root, enter the spinal cord and divide into a long ascending and a short descending branch (Fig. 4). Fibers from caudal-most levels become located medially in the dorsal columns at progressively higher levels, as entering axons are laid down at the lateral margin of the tracts. Thus, the medial bundle of fibers (fasciculus gracilis) originates from segments below the mid-thoracic region while the lateral portion (fasciculus cuneatus) contains fibers from upper thoracic and cervical segments. In addition to the large-diameter fibers entering directly from DRG neurons (primary afferents), the dorsal columns contain many small-diameter primary afferents (conducting under 25 m/sec). Approximately 10% of the dorsal column fibers origi-

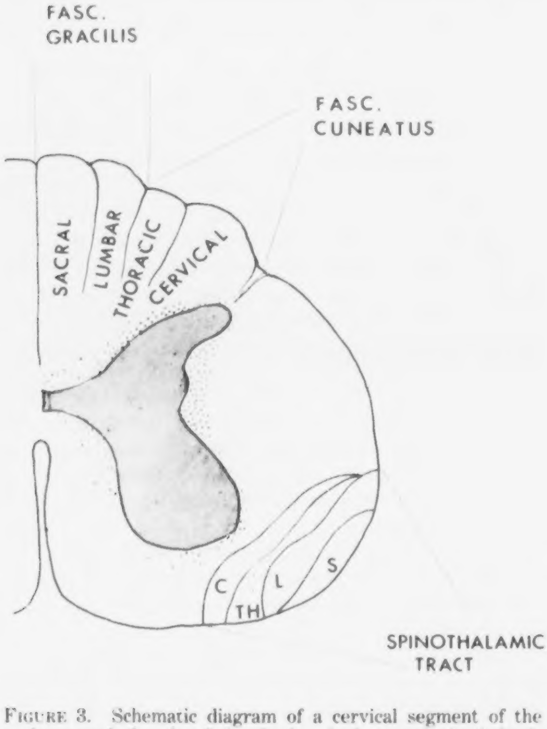


FIGURE 3. Schematic diagram of a cervical segment of the human spinal cord outlining the dorsal column and spinothalamic fiber tracts.

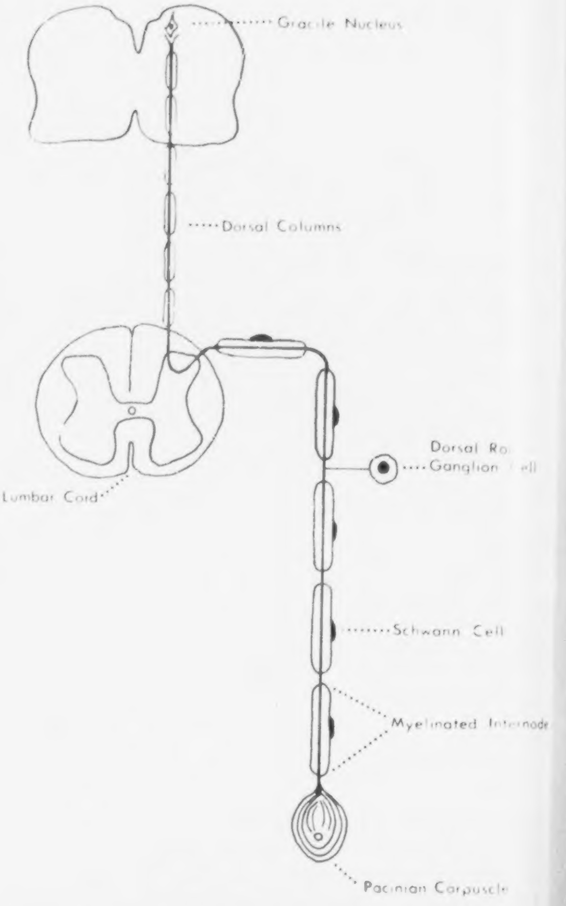


FIGURE 4. Diagram of a dorsal root ganglion cell, depicting the ascending (central) branch of the bipolar axon terminating in the gracile nucleus and the descending (peripheral) branch ending in a Pacinian corpuscle.

nate from cells within the spinal cord (nonprimary afferents). Sensations of touch, vibration and kinesthesia are conveyed by the dorsal columns. The termination of this system is in the ipsilateral dorsal column nuclei of the medulla oblongata.

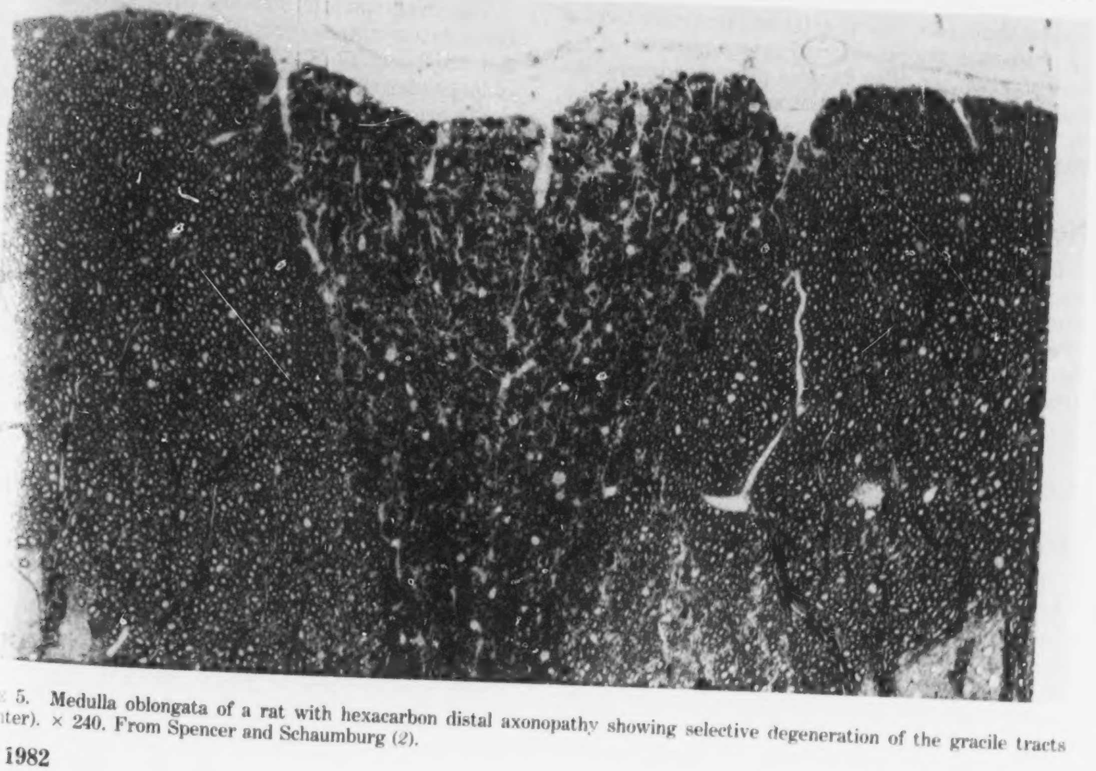
The anterior and lateral spinothalamic tracts convey modalities of pain and temperature, as well as touch. Primary afferents of this system course through the lateral division of the dorsal root and enter the spinal gray matter where they contact several interneurons. Axons of the last neuron in the final relay obliquely cross the cord, within one segment of entry, and ascend in the spinothalamic tracts. There is both topographic and modality-specific organization within the spinothalamic tracts. The most lateral fibers represent the caudal segments of the body, and fibers concerned with pain are, in general, anterior to those conveying temperature. The central projections of the spinothalamic tracts are complex and include several thalamic nuclei (VPL, intralaminar, posterior), as well as reticular nuclei of the brainstem.

The spinocervical tract is a recently discovered pathway responsive to hair movement and skin stimulation. This tract arises entirely within the spinal cord from cells in the dorsal horn of the grey matter. Small fibers of the dorsal root enter the cord and synapse on dorsal-horn neurons. Axons of these cells ascend as spinocervical tract fibers within the dorsolateral fasciculus on the side of root entry, terminating in the lateral cervical nucleus at the uppermost level of the spinal cord. Axons from cells in the lateral cervical nucleus cross the midline and terminate within the VPL nucleus of the thalamus.

A spinoreticular pathway also courses in the ventral quadrant of the spinal cord. The origin of this pathway is complex and largely unknown.

Neurotoxicology

Many of the dorsal-column fibers represent central projections of bipolar DRG cells, and, as described in the previous section, are exquisitely vulnerable in the three common forms of neurotoxic injury. Myelinopathic agents may cause in bubbling of myelin throughout the length of the dorsal columns. Toxins producing distal axonopathy cause degeneration that is initially confined to the rostral ends of the long fibers ascending the gracile fasciculi. The shorter fibers of the cuneate fasciculi are relatively spared in the distal axonopathies (Fig. 5). Neuronopathic agents affect DRG cells at multiple



5. **Medulla oblongata of a rat with hexacarbon distal axonopathy showing selective degeneration of the gracile tracts nter). × 240. From Spencer and Schaumburg (*2*).**

levels resulting in virtually simultaneous axonal degeneration throughout the length of the gracile and cuneate fasciculi.

Other ascending spinal somatosensory pathways are relatively unaffected in neurotoxic conditions. Degeneration in rostral spinocervical axons occasionally occurs in experimental distal axonopathies.

Relay Nuclei

Structure and Function

Neurons in somatosensory relay nuclei, (dorsal column, ventral thalamic, lateral cervical) receive projections from ascending spinal tracts, then project to more rostral structures in the neuraxis. Integration of activity and modification of the ascending volley by corticofugal influences occurs at these sites. Pre- and postsynaptic feedback inhibition characterizes all levels of the somatosensory system and significantly alters the afferent volley.

The dorsal column nuclei (gracile and cuneate) maintain a remarkable degree of topographic and modality specificity. Axons from cells in these nuclei cross as internal arcuate fibers in the low medulla and ascend in the medial lemniscus to the ventral thalamus. The ventral and posterior tiers of the thalamus are subdivided into a number of nuclear aggregates. The ventral posterior lateral nucleus (VPL) is the principal somatosensory relay, and serves as the site of origin for the primary fibers of the thalamo-cortical radiations.

Neurotoxicology

Changes in the dorsal column and lateral cervical nuclei following various types of neurotoxic injury mirror those of the dorsal columns and spinocervical tracts described in the previous section. There are no reports of neurotoxin-induced degeneration in the thalamic relay nuclei.

Cerebral Cortex

Structure and Function

The cerebral cortex controls conscious perception of somatosensory events. The previous simple division of the sensorimotor cortex into precentral (motor) and postcentral (sensory) strips has been replaced by a broad based, multiple mapping of the body surface onto the cortex. Serial and parallel processing of somatosensory information occur at disparate cortical locations and involve extensive interneuron pools, making it difficult to localize the site of action of neurotoxic agents on cortical activity.

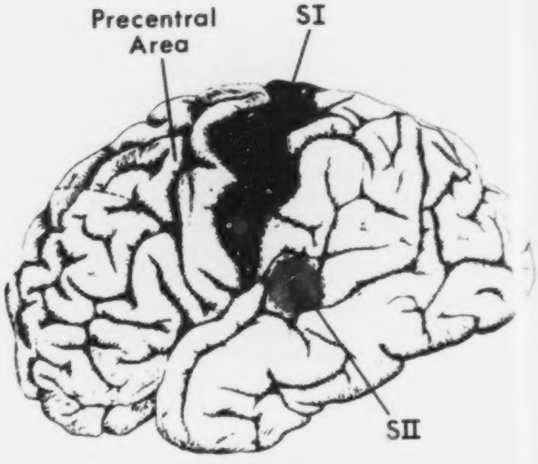


FIGURE 6. Lateral view of the human cerebral cortex outlining the location of the SI, SII and precentral somatosensory regions. Additional somatosensory areas can be found on the medial surface and within the banks of the central and Sylvian tissues.

The first somatic sensory area (SI) comprises three cytoarchitectonic subfields, Brodmann's areas 3, 1 and 2 (Fig. 6). Area 3 receives a dense projection of large fibers from the ventral tier of the thalamus, while areas 1 and 2 receive less dense input of fine fibers from this region. Area 3 and the anterior portion of area 1 principally process short-latency cutaneous and muscle-spindle afferents, whereas the posterior portion of area 1 and all of area 2 receive inputs from deep lying receptors. There are at least two independent mirror-image representations of body surface within SI. For each body representation the caudal dermatomes are represented medially; progressively rostral regions project to more lateral cortical areas. The extent of cortical representation of a body region is proportioned to the use of that region, and not to its size. Thus, in the sensory homunculus, the thumb area exceeds that of the remainder of the arm.

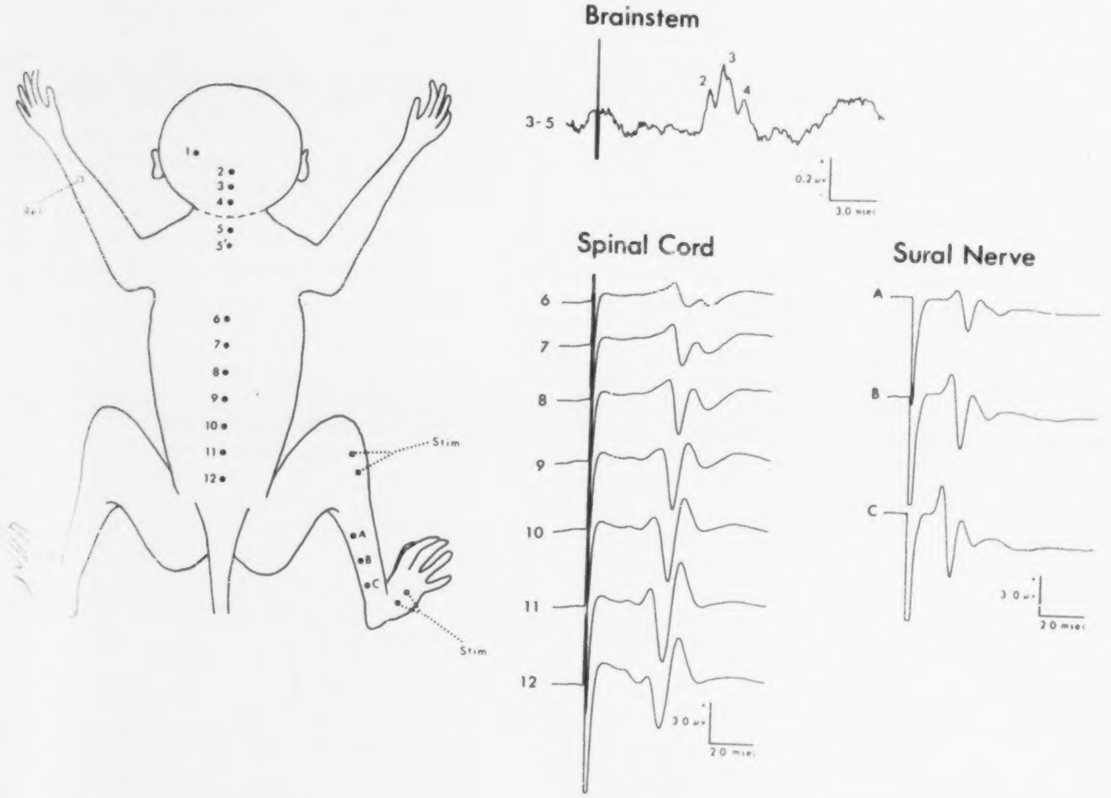
Electrophysiological studies have documented short-latency somatosensory activity within the cortex forming the precentral gyrus (*10*). Independent, direct projections from the ventral thalamus can be traced to this region, terminating within the anterior bank of the central sulcus (area 4). Additional cortical somatosensory regions include the superior and inferior parietal lobule, the SII region within the Sylvian fissure, the retroinsular field and polysensory frontal cortex.

N rotoxicology

I s clear that many neurotoxicants diffusely
aff the cerebral cortex by provoking a variety of
mo ological, pharmacological and physiological
rea ns. This is especially common following acute
ex re to these agents. None appears selectively
to i lve the somatosensory cortex. Similarly, among
sul nces that produce CNS degeneration only
aft rolonged or subacute exposure, few predom-
ina affect the somatosensory cortex. However,
me lmercury frequently produces widespread,
sel ive degeneration of small neurons in the pre-
and ostcentral gyrus, with only minor involvement of
the egions of the frontal and parietal lobe. Mer-
cur also affects small neurons of the calcarine
cort x granule cell layer of the cerebellum and
dorsal root ganglion cells.

Monitoring of the Somatosensory System: Relevance for Neurotoxicology

A salient feature of the somatosensory system is the remarkable length and heavy myelination of its lower conduction pathways. The length of the lower pathways allows noninvasive physiological monitoring at several points. For example, human sensory nerve and spinal-cord conduction velocity measurements are routine clinical procedures. Recent advances in computer-averaged electrophysiological procedures enable the noninvasive recording of activity within somatosensory fiber tracts of higher levels of the CNS in man and experimental animals (*11, 12*) (Fig. 7). Somatosensory-evoked potentials are currently being applied to trace the onset of



Fi 7. Averaged somatosensory evoked potentials recorded overlying sural nerve, cauda equina, spinal cord and brainstem. vity was elicited by a suprathreshold electric shock delivered to the sural nerve.

acrylamide-induced distal axonopathy in the primate (*12*). Initial dysfunction appears at the distal extreme of the gracile component of the dorsal-column system. The change consists of a subtle latency shift (approximately 100 μsec), precedes any behavioral alterations and appears coincident with the earliest detectable morphological change in this nucleus. The sensitivity of this measure appears established, and current studies are focusing on changes in the somatosensory-evoked potential following prolonged low-level acrylamide intoxication and on the ability of the CNS to recover from toxic distal axonopathy.

The authors thank Ms. Laurell Edwards and Ms. Linda Murphy for secretarial assistance and Jim Barna, Monica Bischoff and Kathy Ederle for preparing the illustrations.

This work was supported by MH 06723 and USPHS grants OH 00535, OH 00851, NS 00356, and ES 02168.

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Environmental Health Perspectives
Vol. 44, pp. 31-34, 1982

Embryology of the Eye

by Richard M. Hoar*

A brief description of the basic patterns of mammalian development of the eye is presented based on events as they occur in human beings. The emphasis is not on the details of this development, but rather on its organization and timing, with a figure of comparative development providing a comparison of similar events in man, rat, mouse, and chick.

To an individual struggling to understand some basic mechanisms associated with normal embryology, the development of the eye is well worth study. Here induction has been clearly established as an essential mechanism, and one can discover examples of both primary and secondary inductive forces. Here, too, one finds examples of early developmental "steps" or events whose persistence (or absence) results in either congenital abnormalities or future "disease states." In this system one encounters examples of those unexplained "spontaneous events" which are so common in an embryologist's lexicon. An examination of the development of the eye will serve to remind us that embryogenesis is a continuum. Although discursive presentations such as this tend to emphasize the pieces rather than the puzzle, one should not lose sight of the whole when discussing its parts, for each event we shall discuss is part of an interdependent continuum which can be totally disrupted by the failure of any one of its components either to appear on time or to develop normally. Finally, the development of the eye serves to remind us that there are many instances in normal embryology in which development continues after birth and can be significantly altered by events in postnatal life. The presentation which follows is intended only as an outline of the development of the human eye. For those who wish to pursue to subject, a list of references has been supplied, and a figure of comparative development has been provided to remind you how rapidly these events occur in rodents as compared to man.

Now let us turn out attention specifically to the development of the eye. We see the optic primordia first in man on about day 22 as bilateral evaginations of the neuroectoderm of the forebrain (prosencephalon) which still remains open as bilateral neural folds. These evaginations, the optic peduncles, continue to proliferate laterally as the forebrain closes so that at about day 27 they have become large, single-layered vesicles, the primary optic vesicles, which are continuous with the third ventricle. As they reach the surface ectoderm, these hollow balls of neuroectoderm, connected to the brain through hollow optic stalks, induce the formation of lens primordia, the lens placodes. This is the classical example generally offered as evidence for primary induction in normal development for, unless the optic vesicle reaches the surface ectoderm, no lens vesicle will develop. A groove appears on the inferior surface of each optic stalk and optic vesicle at about 29 days. This retinal (choroid or optic) fissure incorporates both mesenchyme and the hyaloid artery and vein by 33 days, vascularizing the optic stalk and the optic vesicle which itself is undergoing further structural modifications. The hollow ball (vesicle) has become indented creating the appearance of a cup with two layers, an internal and external retinal layer, separated by an intraretinal space that is still continuous through the optic stalk with the third ventricle.

The differentiation of this secondary optic vesicle continues with the fusion of the retinal layers and the disappearance of the intraretinal space except for anterior and posterior cul-de-sacs located at the ora serrata and the intraocular portion of the optic nerve, respectively. The external retinal layer becomes the pigmented layer of the retina by the sixth week and the internal layer differentiates into the neural

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layer, modification of which becomes obvious by 40 days and continues through the seventh month; one can visualize the definitive retinal pattern at approximately 175 days. The changes which occur in the neural layer are similar to those seen as the brain itself develops. At about 40 days, an internal germinal epithelial or ependymal layer may be found next to the pigmented layer, still separated from it by an occasional remnant of the intraretinal space. By 130 days, the ependymal layer gives rise to an external neuroblastic layer, an internal neuroblastic layer and a ganglionic cell layer. By about 175 days, the internal neuroblastic layer has differentiated into the interneuron or bipolar cellular layer. Rudimentary neurosensory rods and cones appear during the twelfth week and continue development after birth. Thus, because of the invagination of the primary optic vesicle to form the optic cup, the neural layer of the retina is inverted and the light sensitive rods and cones are located adjacent to the pigmented epithelium so that light must cross the neural retina to reach the receptors.

In man, from day 35 to day 210 of gestation, the optic stalk has undergone a parallel modification, creating an external layer continuous with the external retinal layer, thus providing the axons from the neural retina a pathway for easy access to the brain. By about the ninth week, the hyaloid vessels are incorporated within the optic stalk and as it fills with axons, the retinal fissure fuses (sixth week); continuity with the intraretinal space and the third ventricle disappears and the hyaloid vessels become the central retinal vessels.

By turning our attention now to the anterior region of the secondary optic vesicle, we can consider the development of the iris, ciliary bodies, lens, vitreous body and aqueous chamber of the eye. As the optic vesicle doubles back on itself (invaginates) forming the optic cup and an inner and outer retinal layer, the point where the two layers meet anteriorly tends to extend over the developing lens to form a pupillary opening. These two layers in front of the lens will become the iris, a process which commences about the seventh week. The internal retinal layer (the neural layer) remains thin in this location and the external layer provides a pigmented epithelium. I wonder what determines the failure of the neural retina in this area to thicken and undergo cell division as it did on the posterior surface of the cup? Could it be the presence of the lens?

The ciliary bodies appear on the inner surface of the developing iris during the ninth week and begin secreting aqueous humor. It should be noted that this system develops from neuroectoderm and functions in a manner similar to that of the choroid plexus.

The lens itself may now be seen as a lens vesicle (hollow ball) having lost its connection to the surface ectoderm during the sixth week. The inner cells of the lens vesicle multiply and extend primary lens fibers towards the opposite external cells, gradually occluding the lumen of the lens vesicle. By the seventh week, the fibers extend between the walls of the vesicle, filling it; forming the nucleus of the lens. New fibers arise throughout life from cells positioned in the equatorial plane. The equatorial plane is located near the origin of the aqueous humor which will become its principal source of nutrients after the hyaloid vessels disappear. Loose bundles of fibers derived from the vitreous framework appear between the ciliary body and the lens forming the suspensory ligament (zonule of Zinn) of the lens.

Posterior to the lens, in an area surrounded by the developing retina, in the lentiretinal space, the primary vitreous body is developing from mesenchyme that infiltrated through the retinal fissure. The retinal fissure begins to close at about 37 days, creating a circular optic cup with no inferior gap, and completely encloses the primary vitreous body by about 47 days. The primary vitreous body and the rapidly developing lens are both supplied by the hyaloid artery. However, the intravesicular vessels degenerate, and the mesenchyme of the primary vitreous body becomes an acellular jelly, the secondary vitreous body, which fills the optic cup behind the lens—a process which is completed about the ninth week. The hyaloid vessels within the optic stalk do not degenerate, but remain as the central retinal artery and vein.

The aqueous chamber of the eye develops during the seventh week between the cornea and iris (anterior chamber) and between the iris and the lens (posterior chamber) under the influence of the lens. The anterior chamber develops as a space in the mesenchyme located between the developing lens and the overlying ectoderm. This space separates the mesenchyme into two layers, a thick outer layer which will become the cornea and a spidery combination of mesenchyme and developing choroid (the iridopupillary membrane) which covers the pupil but regresses and disappears before birth. The fluid medium of the two chambers, the aqueous humor, is produced by the ciliary body and is drained by a large vessel, the canal of Schlemm, located in the iridocorneoscleral angle, the drainage angle or angular sinus.

This complex structure which has formed between the third and ninth weeks of gestation needs protective layers and muscles. Responding to the inductive forces of the pigmented outer layer of the optic cup during the sixth to seventh week, the surrounding mesenchyme forms a vascularized pigmented layer, the choroid (which is analagous to the [illegible]-arachnoid layer of the brain) and a tough white collagenous sclera which surrounds the optic cup with tissue that is continuous with the dura mater of the brain. The cornea which is continuous with the sclera is created from mesenchyme (vide supra) which invades between the developing lens and the surface ectoderm and is covered with a multilayered epthelium anteriorly and a single-layered "endothelium" posteriorly. Under the influence of the lens, the cornea and the covering ectoderm (epithelium) become clear. The extrinsic ocular muscles and the ciliary muscles which control convergence of the lens are established *in situ* from mesenchyme between the fourth to tenth week. The dilator and constrictor muscles of the iris are of neuroectodermal origin, for they develop *in situ* from the pigmented layer of the iris, which was the external retinal layer.

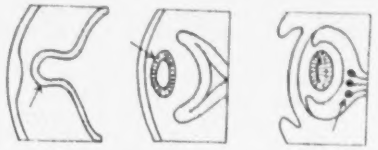
The eyelids appear during the sixth week as folds of ectodermal tissue with a mesenchymal core. They grow until they meet and fuse, during the ninth week, obliterating the palpebral opening, and they remain joined until about the seventh month. Upon fusion of the eyelids, a closed conjunctival sac is formed between the eyelids and the cornea. The lacrimal gland primordia begins to develop from the surface ectoderm in the upper lateral part (superior fornix) of the conjunctival sac during the seventh week. The morphogenesis of the eyelids and lacrimal apparatus is said to be independent of that of the eye, but there is no question that the "wetting action" of the eyelid is absolutely essential to the integrity of the cornea.

Having considered the development of the eye during its major period of organogenesis, between the third and ninth week of gestation, one should remember that development of the eye continues into postnatal life. The fovea centralis of the retina is [illegible] differentiated until 4 months after birth. The [illegible] which, after appearing during the twelfth week, remain poorly developed until about 4 months after birth. The dilator pupillae muscle continues its development until the fifth year of life, and the lens keeps adding fibers to its circumference throughout life at a pace which decreases with age.

I believe that a discussion of this vital system should include commentary on the origin of a few representative birth defects. Coloboma iridis is the result of failure of the retinal fissure to completely close during the seventh week. We now know why a [illegible] in the iris may be indicative of damage to such structures as the ciliary body, the retina, or the optic nerve. A persistent iridopupillary membrane, on the other hand, need not be accompanied by a plethora of other defects, for its development appears to be an isolated event. Although the pigmented epithelium of the retina, the internal retinal layer, becomes firmly attached to the surrounding choroid layer, the retina itself may be separated from the pigmented layer along the lines of that fusion plane which was the intraretinal space. This separation may be assisted by the presence of a persistent hyaloid artery or vitreoretinal adhesions.

Do not suppose that this presentation has more than scratched the surface of the intricacies of the developing eye. However, I hope it has provided you with the stimulus necessary to pursue your investigation of its complexities. To further your efforts, a figure of ocular development has been provided (Fig. 1) comparing the approximate timing of a particular event in the rat, mouse, and man, giving the age in days based on fertilization age, and in the chick according to its incubation age, also in days. In addition, some general references (*1-11*), which should aid your study of the development of the eye, are appended.

DEVELOPMENT OF THE EYE



	Optic Vesicle Forming	Lens Separated	Optic Nerve Fibers Present
Man	24	35	48
Rat	10.5	12.5	14
Mouse	9.5	11.5	13
Chick	1.3	2.5	4

FIGURE 1. Selected developmental stages of the ocular system accompanied by the age in days on which a particular event occurs in the rat, mouse and man based on fertilization age and in the chick based on incubation age. Modified from Monie (*8, 9*).

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Environmental Health Perspectives
Vol. 44, pp. 35-45, 1982

Histopathological and Laboratory Assessment of Visual Dysfunction

by Ralph Heywood*

The currently available methods of assessing ocular toxicity are discussed. Manifestations of ocular toxicity are best described clinically; histopathological examination of the eye is beset with problems of preparing the eye for morphological examination. Electron microscopy is essential to look for chemically induced side effects at the cellular level. Mechanisms of ocular toxicity are poorly understood, and the limitation of animal studies in predicting side effects in man must be appreciated.

The concept of chemical damage to the eye, with effects on vision, is one of the sensitive areas of current toxicology. Because of the problems of adverse reactions it is now required that new chemicals are tested for safety before their general release. Animal studies are essentially comparative, in that spontaneous and induced pathological manifestations are compared in control and treated animals, respectively. The purpose is to identify variations from normal and to define underlying disturbances of physiological mechanisms. The conventional procedure for toxicity studies is to have a control group and three groups of animals given different dose levels of a compound, in order to identify target organ systems and to define no observable effect levels. The regulatory authorities stipulate that these studies must be carried out in a rodent and a nonrodent species. The purpose of this paper is to outline the methods for assessing ocular damage in the commonly used laboratory species, following systemic administration of chemicals, with particular reference to underlying mechanisms of toxicity, and to draw attention to the limitations both in the use of, and understanding of, these approaches to safety evaluation.

The overwhelming number of reports on ocular toxicity (*1-5*) gives the impression that the eye is a potential target organ of every chemical ever discovered. The interpretation of these reports must be kept in perspective.

Examination of the Eye

Ocular toxicity can be monitored by using a variety of techniques: those applicable to animals are listed in Table 1. Because toxicological studies concern groups of animals, the techniques adopted must be simple and applicable to the unanaesthetized animal. Examination of the eye may be by direct or indirect ophthalmoscopy, although indirect ophthalmoscopy is preferred. Slit-lamp biomicroscopy is not recommended in the routine ophthalmoscopic examination. Tear flow can be measured by using commercially available Schirmer tear-test papers. Intra-ocular pressure can be measured either by using the Schiotz' tonometer or preferably, an applanation tonometer.

Functional examination is limited to the testing of the simple reflexes—the fixating reflex, the

Table 1. Examination for ocular toxicity.

- General examination
- Examination with the ophthalmoscope
    - Direct
    - Indirect
- Biomicroscopy
- Schirmer's test
- Ocular pressure
- Functional examination
- Electrodiagnosis—ERG
- Biochemistry
- Pathological examination
    - Light microscopy
    - Electron microscopy

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corneal reflex, the blink reflex and the pupillary light reflex. The fixating and blink reflexes are dependent on the afferent pathways of the optic nerve, optic tract, lateral geniculate body, optic radiation and visual cortex. The efferent pathway is via the superior colliculus, the third, fourth and sixth cranial nerves, and cervical nerves. Constriction of the pupil is dependent on the optic nerve, optic tract, pretectal region with the efferent pathway, the third parasympathetic division, ciliary ganglion and nerve. The consensual response is dependent on decussations in the optic chiasma.

Physiological measurement of retinal integrity can be made by recording electroretinograms. The electroretinogram represents the summated transient electrical activity evoked in response to a light stimulus, of a large number of retinal cells. It is now generally accepted that the outer layers of the retina give rise to the initial negative deflection, or *a* wave, while the activity of the inner layers of the retina is represented by the subsequent positive deflection, or *b* wave. The later positive potential, or *c* wave, is thought to originate in the pigment epithelium. The pattern of response of the rod and cone systems are known to differ, the cones having a higher stimulus threshold than the rods. Brunette and Lafond (*6*) have demonstrated, in rhesus monkeys, that when stimuli of high intensity are used, the rod and cone responses are superimposed, and that a pure rod response is obtained only by using low-intensity stimuli after complete dark adaptation. The validity of the results obtained from an electroretinographic examination relies heavily on the minimization of all artifacts and the standardization of the recording technique. Subtle changes in the electroretinograms recorded during, or after, treatment of experimental animals with novel compounds must be interpreted with caution and assessed in conjunction with other clinical and ultrastructural histopathological findings. Without close correlation with behavioural or morphological evidence, changes in the electroretinogram are not necessarily attributable to disturbance of visual function. The value of the electroretinographic examination in predictive chemical safety evaluations lies in the provision of supplementary evidence to confirm or refute ophthalmological assessments based on conventional clinical and pathological data.

Considerable problems are encountered in preparing eyes for morphological examination. Immediately after the animal has been killed, the eyes should be dissected from the orbits by using the technique described by Saunders and Jubb (*7*) and placed in a fixative. It is essential to use a fixative which will rapidly penetrate the sclera. Zenkers–acetic imposes critical timing but gives good results for the retina. Bouin's fluid is considered to be better for fixing the lens. The fixation, however, most appropriate for routine toxicological laboratories is Davidson's fluid. Formalin fixation inevitably causes separation of the retinal layers in the rat. It is difficult to avoid inducing artifacts in the taking and fixing of eyes. After fixation, the calottes are removed and the eye embedded in fibro-wax. It is useful if the eye can be correctly orientated before sectioning: this can sometimes be done by reference to the ocular muscles and/or by marking the cornea before fixation. Sagittal sections are cut at 5 μm and stained with hematoxylin and eosin. For electron microscopy, the eyes are fixed in sodium cacodylate buffered glutaraldehyde for 5 min. The cornea is then removed and the globe returned to the glutaraldehyde for a further 5 min, after which the iris is removed and the eye allowed to fix for a further 10 min. The lens and vitreous are then removed from the cup, which is allowed to fix for a further 45 min. The retina is subsequently post-fixed in 2% osmium in cacodylate buffer for 1 hr before being dehydrated in alcohol and embedded in epoxy resin.

It is necessary to know the range of normal and spontaneous pathological variations common to the test species. In the dog, rat and primate, many spontaneous abnormalities occur congenitally, or as a result of trauma, infection or developmental and ageing processes.

Manifestations of Ocular Toxicity in Laboratory Animals

The Adnexa

Compounds acting on the adnexa of the eye involve the eyelids or affect lachrymal secretion.

Ptosis of the eyelids, although a common sign in acutely intoxicated rats, is rarely observed following chronic administration of compounds. Edema of the eyelids is occasionally seen, often associated with nonsteroidal anti-inflammatory agent administration. Paracetamol intoxication in the dog induced palpebral edema at the dosage level of 450 mg/kg/day with an associated reduction in the secretory activity of the meibomian glands. The dog has a third eyelid, which is extensive and can cover the whole anterior aspect of the eye; compounds affecting the autonomic system can cause relaxation of this third eyelid.

Conjunctival alterations are most frequently caused by changes in the lachrymal secretion and usually involve the cornea.

The secretion of red tears in the rat, chromodacryorrhea, is mainly associated with stress factors, or an overgrowth of the incisor teeth. Harkness and Ridgway (*8*) have shown that injections of acetylcholine increased almost instantaneously the flow of a dull red secretion, the red color being caused by porphyrins.

Keratoconjunctivitis sicca has been induced in dogs by phenazopyridine hydrochloride (*9*) and some sulfonamides (*10*); the hypotensive agent, clonidine, has recently been found to induce keratoconjunctivitis sicca in the rat (*11*). All anticholinergic compounds given in high dose levels induce keratoconjunctivitis sicca, particularly in the dog. Secretion of lachrymal fluid is not exclusively parasympathetic in origin: stimulation of the sympathetic system also plays a part, and this accounts for the occasional cases of dry eye, seen in dogs given high doses of beta-adrenergic receptor blocking agents.

These conditions of the adnexa are best diagnosed by clinical examination, and, where appropriate, supported by evidence of reduced tear secretion.

Cornea

For a systemically administered compound to affect the cornea it must be secreted in the tears or pass the blood-aqueous barrier. Epithelial erosions are the simplest form for the damage to take, but with continued insult the superficial lesions rapidly can progress to keratitis of varying severity. Some compounds can reach concentrations within the aqueous humor sufficient to damage the corneal endothelium. If damage does occur, the stromal swelling becomes apparent in the whole cornea and the eye appears white and bulges from the orbit. Corneal opacities can be induced in laboratory animals by a variety of compounds, in the rat invariably by morphine (*12*) or the narcotic analgesics (*13*). Lipoidal opacities induced by a nitroaniline have been described in the dog (*14*).

Exacerbation of traumatic lesions and reactivation of old infections can be induced following the administration of steroids.

Toxicity involving the cornea is best diagnosed by clinical examination supported by histopathological examination of the cornea.

Uveal Tract

A side effect of many chemicals is the disturbance of the refractive power of the eye and the ability of the eye to accommodate. The common response of the pupil is dilation or constriction. Dilation of the pupil is induced by the systemic administration of anticholinergic compounds and parasympatholytic agents: constriction is by direct cholinergic or parasympathomimetic action. The effects on the pupil can be modified by central effects.

The high vascularity of the uveal tract should make this a potential site for chemical immunologic reactions. However, this has not been the case, and in my experience one compound only, an antiviral agent, caused sensitization of the uveal tract in a subhuman primate species, with resultant uveitis.

Lens

The lens arises from ectoderm. It is a biconvex transparent body, separating the aqueous and vitreous chambers. It has no blood supply and relies on the aqueous and, to a lesser extent, the vitreous, for its supply of nutrients. The lens continues to grow through life by layering new fibers over the old fibers, so that the old cells become compressed into the center. The lens undergoes a progression of age-related changes and may also develop opacities. The characteristic response to injury is degeneration.

For a compound to be cataractogenic it must traverse the blood-aqueous barrier to enter the anterior chamber. Toxic levels can be achieved only by high daily dosage, slow excretion or prolonged administration. The selective entrance of drugs into the aqueous is dependent on molecular size and lipid solubility. Large molecules are held back by the capillary membrane. Lipid-soluble substances rapidly traverse the blood-aqueous barrier and reach equilibrium.

Chemically induced lenticular lesions are cataracts and can be classified into two major groups: transient lens opacities and permanent cataracts.

Transient Lens Opacities. Opacities of a transient nature, in which the lens returns to normal within a few hours, have been produced in young rats following the administration of substituted phenothiazines, catecholamines and morphine-like analgesics. Transient lens opacities have been produced in young Beagle dogs following the administration of some tranquillizers, some diuretics and diisophenol. These opacities are usually associated with the anterior capsule, where they appear as crescents around the periphery of the lens, or as discrete opacities on the anterior surface of the lens (Fig. 1). These acute reversible lens opacities are ascribed primarily to changes in the osmolality of the aqueous humor, although other factors may be involved.

Permanent Cataracts. The clinical development of permanent chemically induced cataracts follows distinct courses. There is always a latent period between the administration of a compound and the

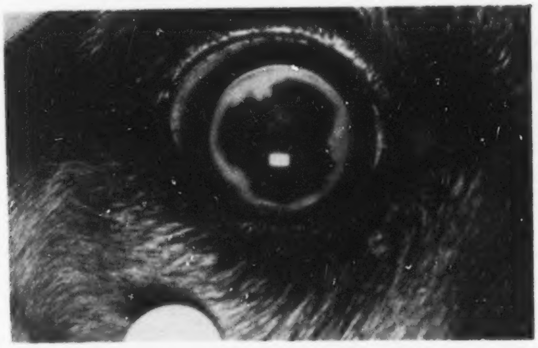


FIGURE 1. Transient lens opacity.

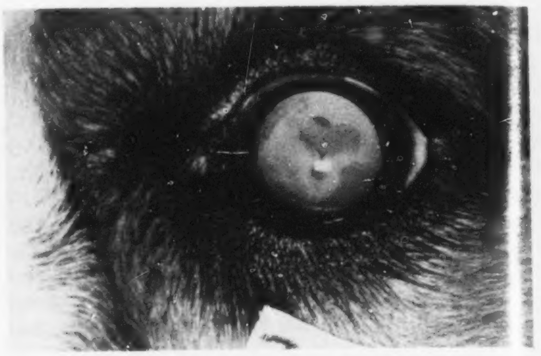


FIGURE 2. Opacification of superficial lens fibers.

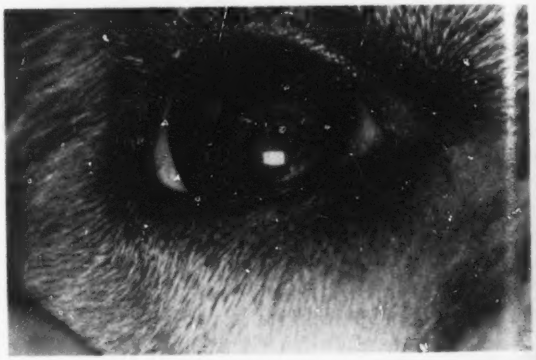


FIGURE 3. Triangular posterior polar subcapsular opacity.

onset of lenticular change, and this can vary from a few days to several months. Many cataracts start at the equator of the lens. The first sign is the appearance of vacuoles and striation around the equator of the lens. The lens at the equator becomes opaque and appears to have a surrounding halo. Opacification of the superficial lens fibers continues and extends under the anterior or, more frequently, the posterior capsule of the lens. Initially, only those lens fibers immediately below the capsule are involved (Fig. 2). The most common form of chemically induced cataract is that which starts at the posterior pole of the lens. In the dog, these cataracts appear roughly triangular at the posterior pole, involving only subcapsular fibers (Fig. 3). In the rat, chemically induced cataracts show a variety of morphological patterns, but often they first show as triangular opacities at the posterior pole. The most likely explanation of the greater involvement of the posterior cortex of the lens in toxic cataracts is because posteriorly the capsule is thinner and epithelial cells are absent, allowing the toxic agent to penetrate into the superficial fibers more readily. At the posterior pole the lens is at its greatest stress with respect to nutrients and oxygen. The only other manifestation of lenticular toxicity that has been shown is that of a changes in refraction. Changes in the transparency as well as the refraction of the lens nucleus have been recorded following the administration of DMSO (*15*) and with *p*-chlorophenylalanine (*16*).

Cataracts are best diagnosed by clinical examination; processing of the lens for histopathological examination is of very limited value. Chemicals with widely differing structures and pharmacological activity have been reported to cause cataracts in laboratory animals. These agents have been reviewed by Gehring (*17*). Such compounds defy easy classification because the mechanisms of their cataractogenicity are poorly understood.

Cataracts can be induced by feeding high levels of galactose and xylose to rats. Animals made diabetic by the administration of alloxan or streptozotocin or those with high dose levels of progestational agents develop cataracts. Here the common mechanism is the formation of sugar alcohols which accumulate; because they are not easily metabolized they cause hypertonicity and osmotic swelling. Those alkylating agents that are cytotoxic induce cataracts by interfering with cell proliferation. Triparanol, a drug once used in the treatment of hypercholesterolaemia, induces cataracts in the rat and the dog. The opacities appear as peripheral striate opacities, and sudanophilic material has been identified within the lens fibers. It is possible that this type of cataract is a manifestation of phospholipidosis. The cataracts induced by other compounds, such as the sulfonylurea drugs, the chelating agent desferrioxamine, sulfonamides, the steroids such as meth ibure and clomiphene citrate, and chemicals such liquat, heptachlor and mirex, are of unknown mech isms. Many of the agents inducing cataracts may so by specific interference with enzyme syst . The toxic effects of various substances on the l are quantitatively very different in different s ies.

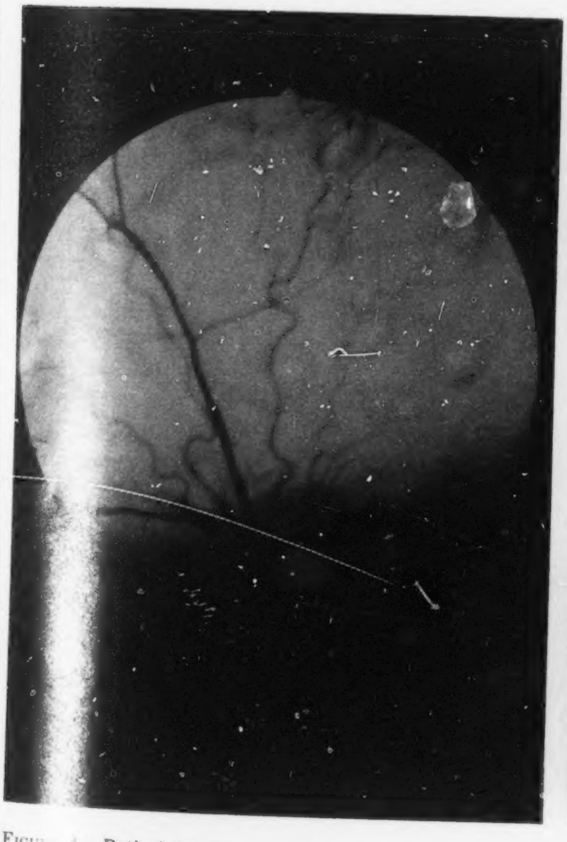
Reti a

Th esponse of the ocular fundus to chemical insul an be classified into three main categories: hem hage; the presence of exudates; and degenerati changes.

Co pounds which cause hemorrhage do so by actin directly or indirectly on the clotting mechanism. he blood frequently penetrates the hyaloid memb ane and appears in the vitreous as blobs or clots.

Exudates are caused by the escape of plasma and/or white blood cells from defective blood vessels. Exudates between the pigment epithelium layer and the rod and cone layer can cause partial detachment of the retina (Fig. 4). The quinines, such as imidazoquinazoline and quinine sulfate, can induce this type of change in the dog (*18*). Occasionally, the compound or a metabolite of the compound can be found to leak from retinal vessels.

The drug-induced retinopathies have been reviewed by Meier-Ruge (*19*), with the main discussion directed towards the antimalarial chloroquine, and the psychotropic phenothiazine derivatives. Of the three categories of phenothiazines, only the piperidines induce retinal lesions. Of these compounds, thioridazine has been most extensively studied. Retinal lesions can be readily induced with these compounds in the cat and dog; on examination, the retina of the tapetum has a coarse spotted appearance (Fig. 5). The lesions first occur in the primary visual cells, with secondary changes in the pigment epithelium.



FIGU 4. Retinal detachment: dog.

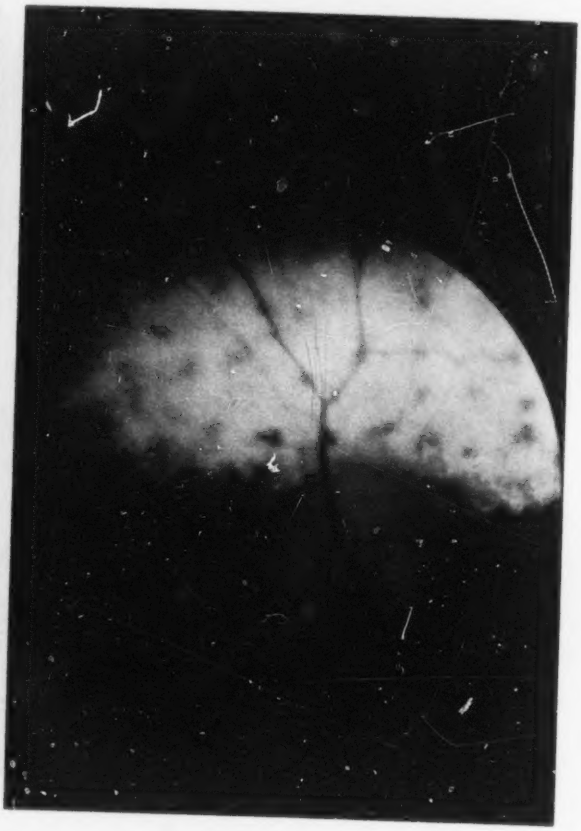


FIGURE 5. Thioridazine toxicity: dog.

Meier-Ruge (*20*) has shown by histochemical techniques that there is a massive loss of enzyme activity in the ellipsoids of the rods. He is also of the opinion that the absorbent binding to melanin is of importance.

Experimental chloroquine retinopathy has caused considerable controversy over the mechanisms of the toxicity. It is certain that chloroquine binds to the pigmented tissues of the eye (*19*, *21-23*), but it is doubtful whether the pigment binding is of relevance to the primary retinopathy. From work carried out in the albino rat and pigmented strains, rabbits, beagle dogs, cats and the rhesus monkey, the initial reaction of the retina is the formatio of membranous cytoplasmic bodies (myelinoid bodi s), and these myelinoid bodies can be induced wi hin one week of starting treatment (Fig. 6). Rosen hal et al. (*23*) clearly demonstrated that degenera ve changes in the eye of the monkey occur in he nucleus and cell body of both the rods and cones nd that the pigment epithelial damage was a l ter manifestation of chloroquine toxicity. In this ex eriment in the rhesus monkey, very extensive d mage occurred in the retina, although there wa no clinical or functional evidence of change.

The binding capacity of the pigment tissues o the

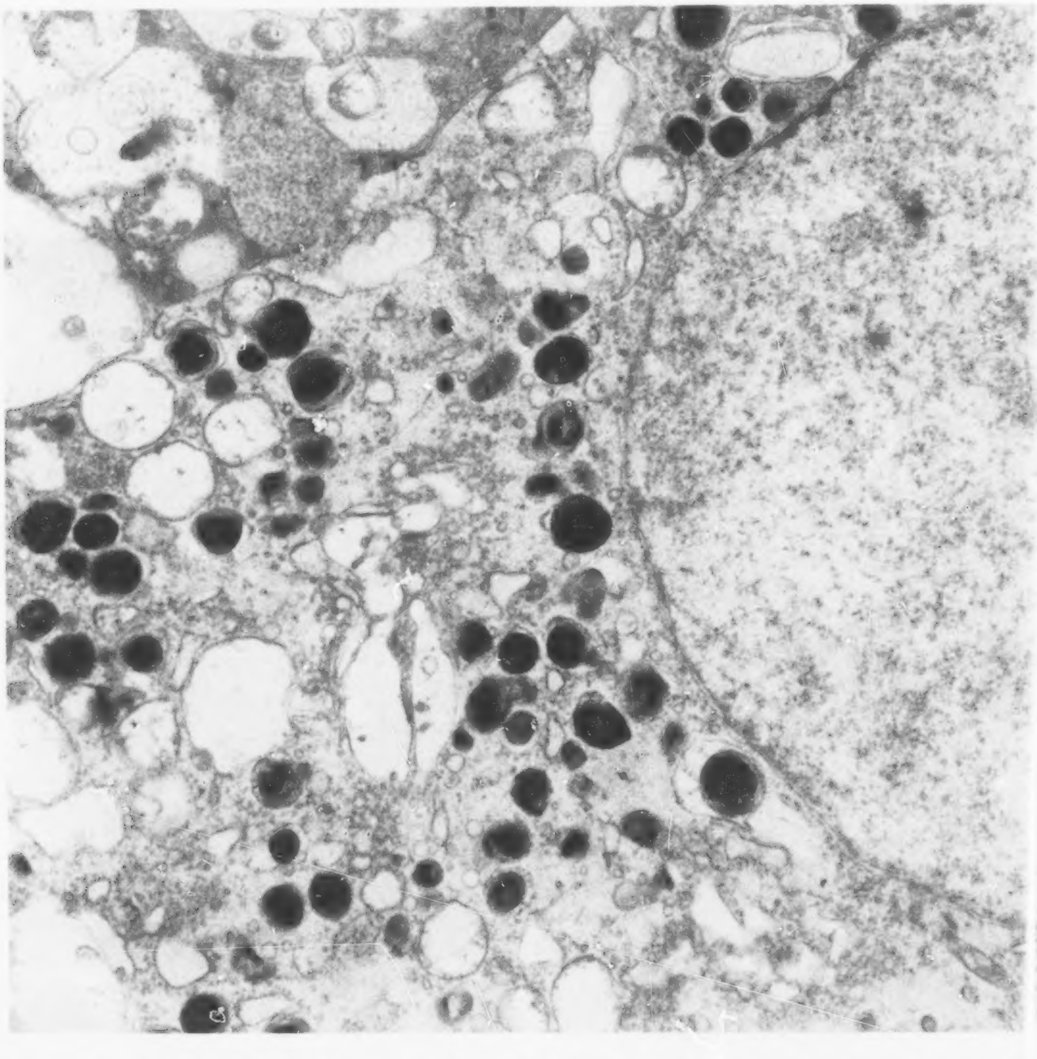


FIGURE 6. Myelinoid bodies. Ganglion cells of rat retina following 5 doses of 100 mg/kg/day of chloroquine. × 14,30

eye have been demonstrated for many compounds besides chloroquine and the phenothiazines. The beta-blockers, rifampicin, many antiprotozoals, tetracyclines, glycosides and most polycyclic compounds bind to melanin. Compound uptake apparently increases with time, and once bound to melanin it can be retained for long periods. With the majority of compounds there is no resultant retinal toxicity, presumably because the compound is bound in an inactive form. In some cases, as with some phenothiazines and chloroquine, once the binding capacity has been exceeded, direct damage may result.

There are few reports of drug-induced retinal toxicity in the rat. Cyproximide, a psychotropic agent (*24*), a propionic acid derivative (*25*) and a nitrosourea (*26*) have been recorded as inducing retinopathies in the rat.

The administration of amphophilic compounds to the rat results in the formation of myelinoid bodies in many cell types. These myelinoid bodies are uni- or multicentric. They are particles limited by a single membrane and containing osmiophilic membranes in concentric arrangement.

The formation of myelinoid bodies is a manifestation of chemically induced lysosomal storage disease. Generalized phospholipidosis has been induced by a variety of chemical agents which include antihistamines, hypolipidemics, anti-inflammatories, antidepressants, anorectics and coronary vasodilators (*27, 28*). In chemical safety evaluation studies it has

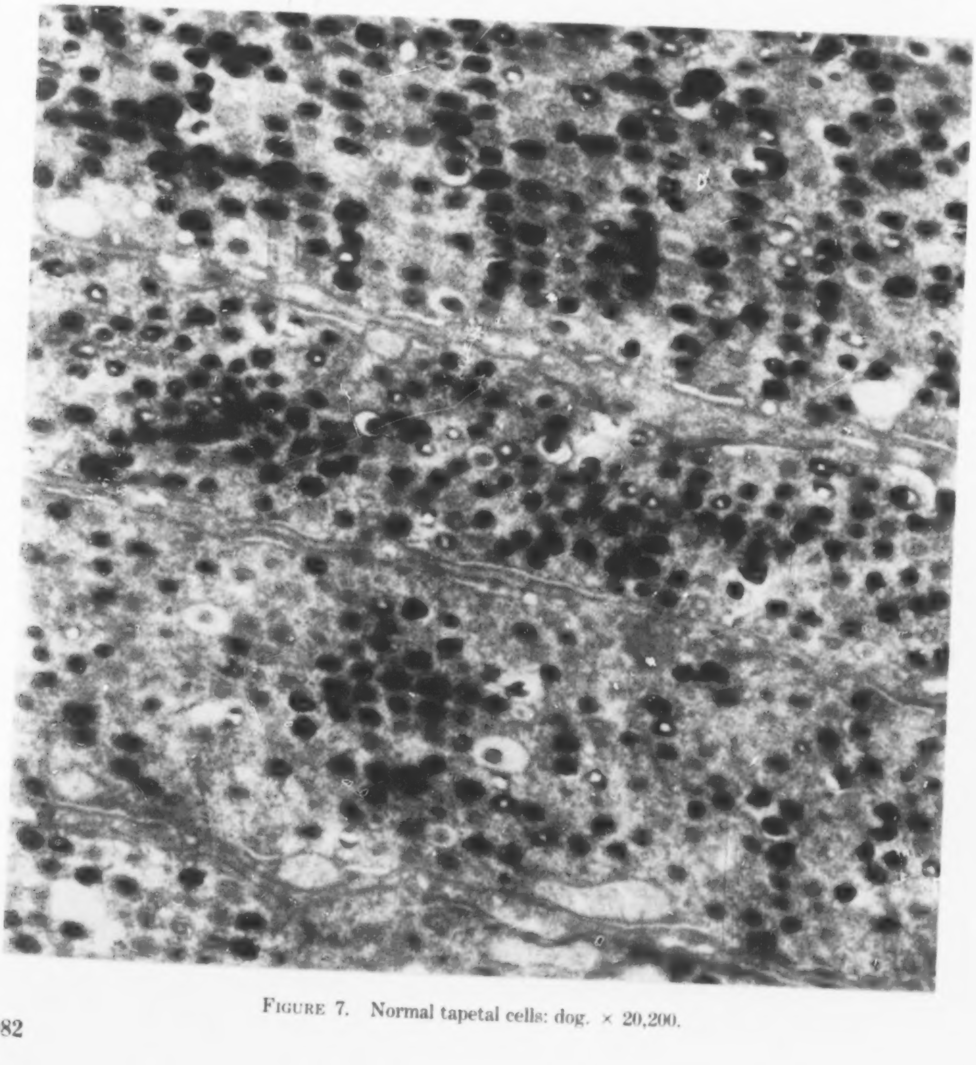


FIGURE 7. Normal tapetal cells: dog. × 20,200.

been the drug-induced pulmonary lipidosis with the accumulation of foamy macrophages which has promoted interest. Drenckhahn and Lullmann-Rauch (*29*) have focused attention on drug-induced retinal lipidosis, which has been caused by several compounds. Myelinoid bodies have been found in the inner retinal cell types and the pigment epithelium. Different compounds have given different distributions: some compounds, like triparanol, have shown a predeliction for pigment epithelial cells, while chloroquine affects mainly the ganglion cells. Some compounds which have been shown to produce a marked generalized phospholipidosis involving lungs, liver and endocrine tissues, peripheral and central nerve cells, and epididymal tissue, have induced only a mild degree of lipidosis in the retina. It seems that the accummulation of myelinoid bodies within the cell does not lead to cell death, and there is some evidence that, even with the continued administration of compounds, the cells recover from the initial shock, and the accumulated myelinoid is not progressive. Myelinoid bodies tend to disappear after cessation of treatment. The use of electron microscopy is essential to look for these myelinoid bodies. Myelin figures can be produced in tissues fixed in glutaraldehyde, if fixation is prolonged. These figures could be mistaken for pathological change.

Some chemicals cause a loss of tapetal color. The change first appears as patchy fading of the tapetal color, until eventually the entire tapetum has a bleached appearance. No histological change can be

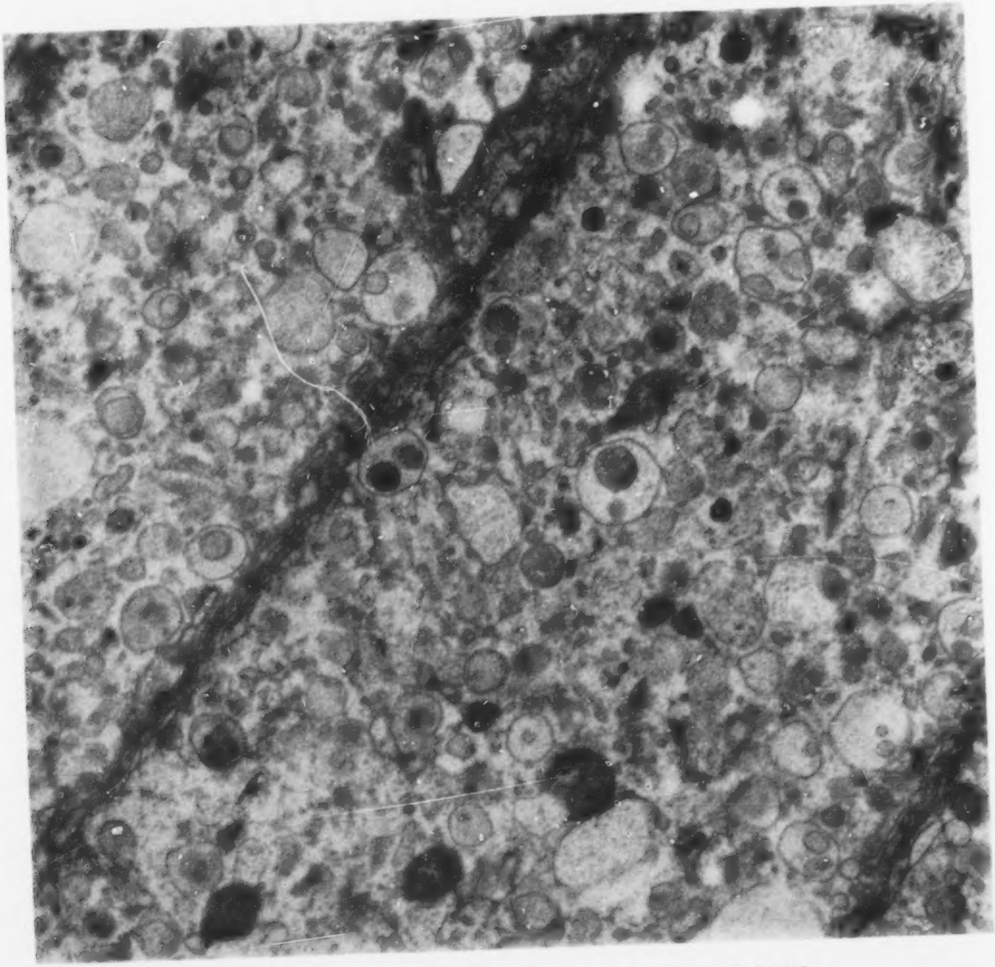


FIGURE 8. Tapetal cells for animals with a bleached tapeta. × 20,200.

detec by light microscopy, but electron microscopy ws the presence of ultrastructural changes. T tal cells from normal dogs are characterized b he presence of parallel groups of uniformly dense tracytoplasmic rods (Fig. 7): in animals with ached tapeta the parallel arrangement of the r is disrupted, the rods are surrounded by vacu which are not uniformly dense, appearing swoll with indistinct outlines (Fig. 8). No functiona eration can be detected by ERG other than slight olongation of the implicit time of the wave forms oked in response to low-intensity stimuli in the d -adapted state. These changes are attributed the chelating action of these compounds: ethambutol is one such compound (*30*).

Clinical and functional examinations of the retina are shown to have considerable limitations in detecting toxicity. Histological examination of thick sections will detect only the most gross lesion. The use of electron microscopy is essential to look for chemically induced side effects at the cellular level.

Optic Nerve

The response of the optic nerve is limited: it may show atrophy or it may increase in size. The cases of atrophy show as a reduction of myelinated axons or demyelination of the optic nerve. In the dog, an organophosphate pesticide—ethylthiometon (*31*)—and clioquinol (*32*) induce this type of change. Ethambutol has caused demyelination of the optic nerve, chiasm and tract in the monkey (*33*) and the rat (*34*).

Edema of the disc is reported in monkeys with methyl alcohol poisoning (*35*) and trimethyltin acetate ( ). Salicylanilide (*37*) causes vacuolation of the cerebral white matter in the dog and edema of the di Fig. 9), and hexachlorophene (*38*) induces perip ary exudations in the dog.

Ch s in the optic nerve are readily diagnosed by th ndard clinical and histopathological methods, ugh impairment of the pupillary light refl requently the first evidence of damage.

Dis sion

Th lidity of safety evaluations is based on the assu on that extrapolation from animal to man is p e. It is appropriate to ask how predictive are common laboratory animal species with resp o ocular toxicity.

In ecent survey, in which the toxicological prof f 50 compounds were compared in rodent and rodent species, Keywood (*39*) found that in the , 26% of the compounds and, in the monkey spec , 7% of the compounds, induced some form of ocular toxicity, whereas in the rodent, none of the compounds affected the eye. A review of the literature shows some correlations for ocular toxicity between the rodent and nonrodent species—for example, triparanol, some sulfonamides, chloroquine, iminodipropionitrile—but such correlations are rare.

If we try to compare adverse eye reactions in man with toxic findings in animals, again the correlations are poor. The classical cataractogenic agent cited for man is dinitrophenol. Animal models to investigate the cataractogenicity of this drug have been.the duck or chicken, special strains of mice, scorbutic guinea pigs, rabbits under 1 month, or 3-week old dogs. It is apparent that current safety evaluation studies in rats and beagle dogs or nonhuman primates would not have predicted the cataractogenic potential of this compound to man. Cortical posterior subcapsular cataracts have been induced in man, both in children and adults, by a variety of glucocorticoids. Animal experiments have failed to induce changes in the lens. The keratoconjunctivitis sicca described by Wright (*40*) as part of

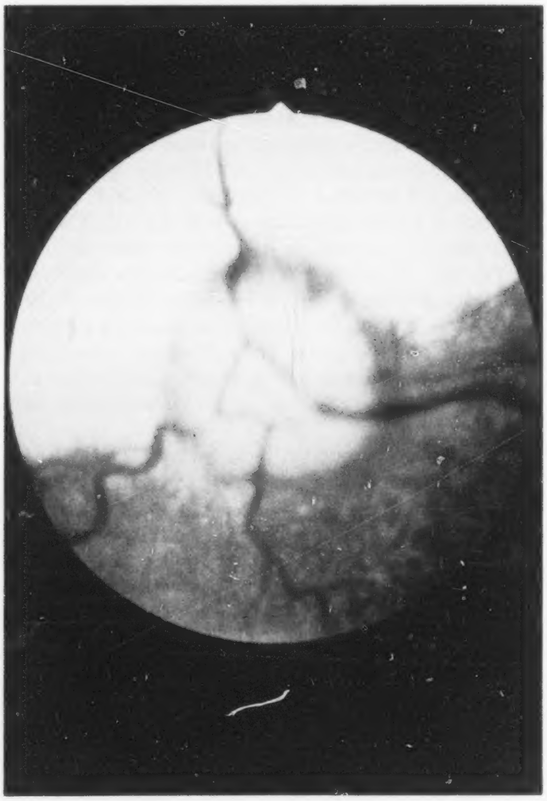


FIGURE 9. Edema of the disc: dog.

the mucocutaneous syndrome induced by practolol, was not predicted by conventional animal experimentation. Attempts to reproduce subacute myelo-optic neuropathy (SMON) in experimental animals has yielded unreliable and sometimes equivocal results. Worden and Heywood (*41*) drew attention to the fact that the lesion induced in the dog did not accord with the criteria for SMON in man.

Some findings in animals, have not been confirmed by human clinical experience. In aging monkeys, both controls and particularly animals undergoing long-term administration with progestogens, hyperfluorescent spots around the macula have been described. Fine and Kwapien (*42*) have shown that these lesions consist almost exclusively of lipoid degeneration of the pigment epithelial cells. The significance of this finding in relation to women using oral contraceptives is not known; however, there is no clinical evidence to establish an adverse effect of oral contraceptives on the retina. From the data presented it would appear that all amphophilic cationic compounds have the potential to affect the retina in the rat, and form myelinoid bodies. The same mechanism is evidently not operant in man.

Many compounds affect physiological processes which may involve the visual system but which cannot be predicted from animal experimentation. Of 56 compounds investigated by Laroche and Laroche (*43*), 34 were found to decrease color discrimination significantly; 18 of these 56 compounds were antibiotics, and nine caused some impairment of vision. Of patients on digitalis, 10–25% experience some visual symptom of toxicity (*4*). The hallucinogens have variable effects on vision (*44*).

The eye may show side effects from a wide variety of drugs used in clinical medicine. Some of these side effects, although undesirable, must be accepted as unavoidable. Many of the ocular side effects are caused by overdosage or indirect consequences of the drugs' primary action, or by an individual idiosyncracy.

Target-organ toxicity, induced by high dose levels of test compound in laboratory animals, is of little importance in the assessment of safety of compounds. Safety evaluation should be based on the no observable effect level. As far as the eye is concerned, animal experiments have been shown to have little predictive value for man.

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Environmental Health Perspectives
Vol. 44, pp. 47-53, 1982

Psychophysical Assessment of Visual Dysfunction

by Patricia M. Blough* and John S. Young†

Selected psychophysical techniques for nonhuman animals are described. These include operant learning methods and a new reflexive technique that may prove especially efficient. Problems of particular interest for toxicological research include control of the physical stimulus, choice of species, separation of stimulus from attentional effects, response bias, and pre- and post-training efficiency.

Animal psychophysics offers a variety of sophisticated tools to the behavioral toxicologist. Available methods permit us to assess sensory functions in nonhuman subjects with remarkable precision; it follows that we can also measure toxin-induced effects on sensory systems. Recent reviews (*1, 2*) survey the techniques of animal psychophysics and consider their problems. In the present paper we shall consider only a few of those methods, selected for their applicability to toxicological research.

The association between stimuli and responses is the basis of most animal psychophysics. In most cases this association is established through training. By using the techniques of operant or classical conditioning, animals can be taught to discriminate among stimuli by associating different responses with different stimuli. The animal psychophysicist starts with an easy discrimination and then varies the stimulus along the desired dimension in order to find a "threshold" or point at which the discrimination breaks down. Figure 1 describes a simple example. Hungry pigeons were rewarded with food for pecking a disk illuminated with light of a particular wavelength. When illumination was provided at any other wavelength, pecks were not reinforced. The function in Figure 1 describes the pigeon's ability to discriminate among wavelengths in each of two spectral regions by showing the probability of incorrect responses to wavelengths near the correct value. The slope of the curve is an index of discriminability. These functions indicate that the birds discriminate better in the 600 nm region than in the 540 nm region of the spectrum. This example illustrates a relatively unsophisticated example of Skinner's operant conditioning methodology. As in most animal psychophysics, reward or reinforcement substitutes for the verbal instructions available in human experiments.

While operant learning methods are most widely used, classical and reflexive techniques may be more appropriate in some instances. Later in this paper we shall illustrate the special applicability of a reflexive technique, and subsequent papers will describe a variety of other psychophysical methods.

In choosing and applying the techniques of animal psychophysics, the particular sensory modality is only one factor. Other considerations include the clear establishment of the stimulus-response relationship and the separation of sensory and nonsensory effects. For toxicological research, efficiency is also an important consideration.

Establishment of the Psychophysical Relationship

A relationship basic to all psychophysics is known as the psychometric function. It describes the variation in a response measure with the variation of a a stimulus along a clearly-defined continuum. Figure 2 illustrates a psychometric function obtained

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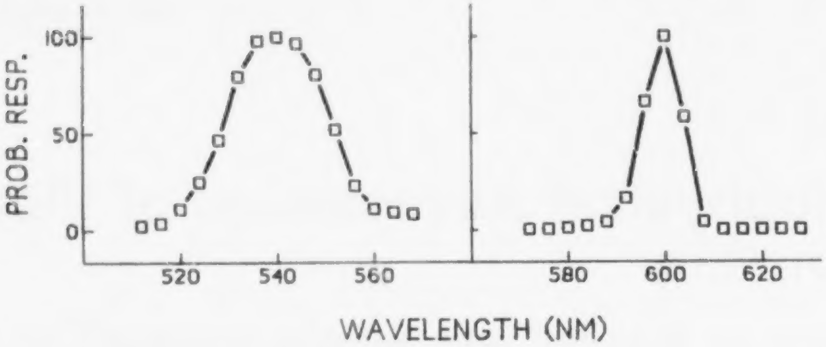

FIGURE 1. Probability of response to a continuum of wavelengths: (left) data from pigeons rewarded only for responding to 540 nm; (right) data from birds rewarded only for responding to 600 nm. Responses to other wavelengths result from the birds' inability to discriminate them from the rewarded value. Thus, the slopes of the two functions reflect wavelength discriminability for two regions of the spectrum.

for pigeons. The response measure was simply the probability of the key peck; the stimulus varied along the dimension of luminance. Reward was available only when the response key was illuminated. On half the trials the key was dark and no responses were reinforced. Thus, this function indicates the bird's ability to detect the presence of lights of varying luminance.

One reason for the establishment of a relationship like this is that drugs and toxins seem to have their clearest effects when external control of behavior is weak (*3*). For example, Evans (*4*) has shown that scopolamine interacts with stimulus discriminability in its effect on performance. To describe the early or subtle effects of toxins, it seems most useful to consider stimuli in the range where discrimination is poor; these would be values in the intermediate portion of the psychometric function. Establishment of the complete function is important, however, because a clear relationship indicates good stimulus control of the response. (Animals can be quite expert at detecting extraneous variables that the experimenter has failed to control.)

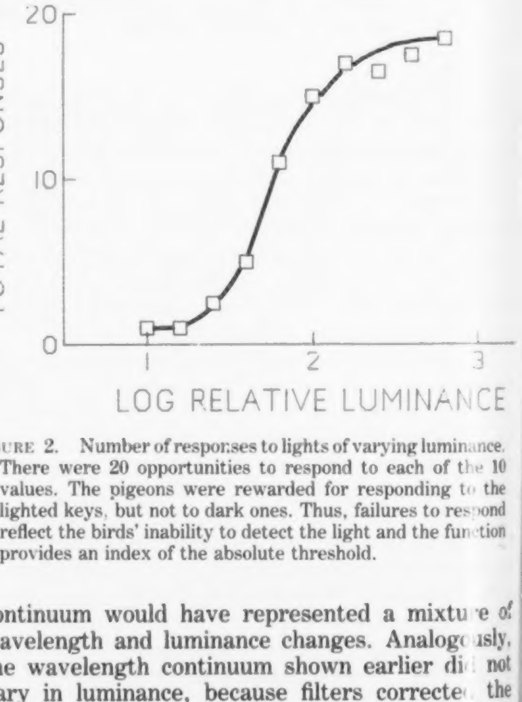

FIGURE 2. Number of responses to lights of varying luminance. There were 20 opportunities to respond to each of the 10 values. The pigeons were rewarded for responding to the lighted keys, but not to dark ones. Thus, failures to respond reflect the birds' inability to detect the light and the function provides an index of the absolute threshold.

Special Problems in Vision Research

The control of the stimulus side of the psychometric function requires special sophistication with regard to the sensory modality under investigation. For example, the stimuli described in Figure 2 varied in luminance alone, because neutral density filters were used to control the energy of the light. Had changes in electrical resistance been used to vary the output of the stimulus lamp, the stimulus continuum would have represented a mixture of wavelength and luminance changes. Analogously, the wavelength continuum shown earlier did not vary in luminance, because filters corrected the lights so that they would have equivalent luminances for the pigeon's eye. These are just two examples of the complex problem of controlling the visual stimulus; technical details are discussed by Boynton (*5*), among others.

A second consideration that specifically pertains to the study of vision concerns the duplexity of the visual system. Subjects working in well-lighted conditions probably use photopic or daylight vision, mediated by the retina's cones and good for tasks requiring acuity or color vision. Subjects who are adapted to darkness use the scotopic visual system, mediated by rods and especially sensitive to dim light. There are other procedures for separating the two systems; for example, the photopic system is relatively more sensitive to long wavelengths and to lights flickering at high speeds. Isolation of photopic and scotopic systems can be helpful in understanding the nature of treatment effects. For example, recent research of Merigan (*6*) has elucidated the effects of methylmercury by varying both adaptation conditions and frequency of a temporally modulated light. His findings, suggesting that the photopic visual system is involved, help to clarify the constriction of the visual field seen after mercury poisoning.

Finally, in using nonhuman subjects for vision research, one must consider the special qualifications of each particular species. This problem is complex. Not only do different species appear to respond differently to toxic agents; there are also differences in certain features of their visual systems. Most mammals, for example, are more dependent on scotopic vision than are humans. The macaque monkey, an exception, has been an excellent subject for visual studies (*4*, *6*). A less expensive species is the pigeon, which has excellent photopic vision. However, we do not yet know how toxic agents affect its visual system and whether there is useful comparability between birds and humans in this respect. It is worth noting here that species differences, when understood, can often serve as tools to help clarify factors affecting vision.

Isolation of Sensory Effects

It is well known that toxic agents affect many aspects of behavior. The assessment of strictly sensory effects requires special precautions to avoid confounding behavioral deficits. Recent research of Hayes (*7*) illustrates this problem. These experiments attempted to separate the sensory effects of certain drugs from their effects on the subject's attention. A drug that leads to an attentional deficit should affect performance in all phases of a discrimination task, regardless of its difficulty. If the effect is strictly sensory, however, we would expect to see the deterioration limited to difficult discriminations.

Hayes' research used a more sophisticated application of the operant method than those seen in our earlier examples. Again, pigeons were trained to discriminate among wavelengths of light. In this case, however, there were two response keys available. The birds were rewarded for pecking the one on the left if the wavelength was shorter than 572 nm; for longer wavelengths they were rewarded for pecking the right-hand key. The functions in Figure 3 illustrate an attentional deficit induced by pentobarbital. As in the earlier examples, the slope of the curve is an index of sensory function; steeper slopes indicate better discriminability or smaller differential thresholds. In contrast, the asymptotes of these curves provide a measure of attention (*8*). If attention were perfect, the left asymptote would be at zero and the right, at 100% correct; that is, the pigeon would never peck the right key when the wavelength was very short, and it would always do so when the wavelength was very long. Hayes' findings indicate that pentobarbital affects both the slopes and the asymptotes of these psychometric functions. Thus, the drug effect on a sensory system was confounded by its effect on an attentional system.

A related problem concerns the separation of response bias from stimulus sensitivity. Suppose, for example, that a certain toxin led to an increased activity level that, for a pigeon, was reflected in an

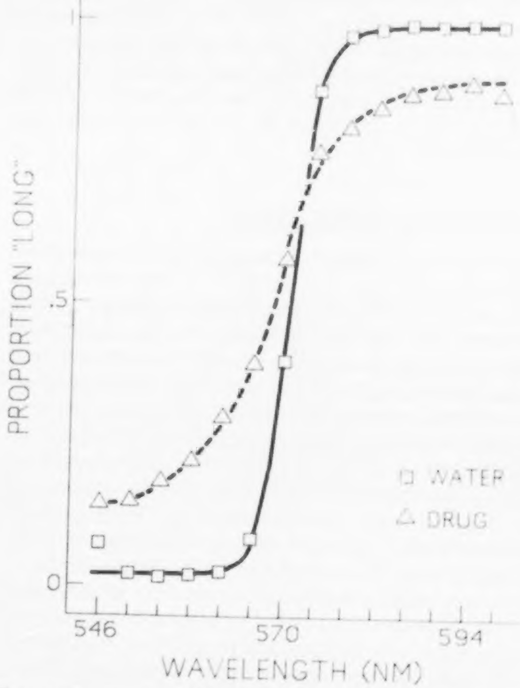

FIGURE 3. Psychometric functions obtained (□) before and (Δ) after pentobarbital treatment with pigeons. Two responses were available. The one shown was correct only when the stimulus wavelength was greater than 572 nm. The drug appeared to reduce attention since treatment led to more errors on easy as well as hard discriminations. Data are from the research of Hayes (7).

increased likelihood of the pecking response. Had the procedure been the "go/no-go" method illustrated in Figure 1, the effect would have been an increase in response probability to all wavelengths and an apparent, but misleading reduction in sensitivity. In fact, there are data (*9*) that do show a selective effect of a drug on bias. The theory of signal detection (*10*) provides us with techniques for coping with this problem. Blough and Blough (*1*) discuss their application to animal psychophysics. When the two-key method used by Hayes is employed, it is possible to evaluate effects on bias as well as those on attention.

Other data, mainly from behavioral pharmacology, should also lead us to be wary in our selection of psychophysical tools. For example, rate measures (number of responses over an extended trial) are prevalent in the operant discrimination literature. Yet a considerable body of data suggests that drugs have complicated effects on response rate (*11*). In some cases, a particular agent may raise an initially low rate, but lower one that is initially high. Thus, rate measures are unsuitable for the assessment of sensory effects. The experiments illustrated in Figures 1, 2 and 3 used trialwise procedures. Each trial began with the presentation of the stimulus and lasted only briefly. The resulting measure, the probability of a particular response during a brief trial, is a more reliable index than the traditional rate measure.

Improving Efficiency

Collection of data such as those illustrated above is notoriously time-consuming. For example, it took 11 weeks to gather the data shown in Figure 3. The necessity for lengthy training is one source of difficulty; animals typically improve over time, and stable pretreatment performance may not be reached for several weeks. A second problem concerns limitations on the amount of data that may be obtained in a single session. In typical operant studies, this limitation is imposed by the amount of the food or water reward that can be consumed without satiating the subject. For certain toxic agents it may be desirable to administer the toxin only once and to maximize the amount of data that can be collected immediately afterward. One way to improve the efficiency of single sessions is to reduce the probability or the amount of reward. When these values are low, satiation occurs less quickly, and a session can consist of more trials. We have found that pigeons will maintain good stimulus control when correct responses are reinforced with a probability as low as one out of 32 occurrences. Thus, the lengthy training required for operant psychophysical methods can pay off with quickly obtained, reliable post-treatment data.

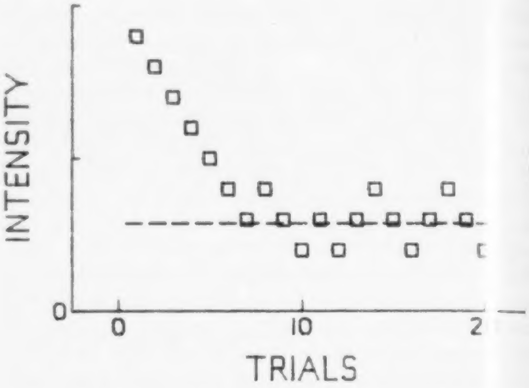

FIGURE 4. Schematic representation of a modified tracking procedure. The subject's behavior on each trial determines stimulus intensity for the following trial. A correct detection decreases intensity; when the subject fails to detect the stimulus, its intensity is increased. The dashed line indicates an intensity that might be taken as absolute threshold.

Another method of improving efficiency in the post-treatment stage is to maximize presentations of test stimuli in the region of the threshold. A useful set of methods, known as "tracking" (*12*), uses feedback from the subject to determine stimulus values. The stimulus initially diminishes in strength until the response measure indicates that the subject no longer detects it. Strength is then increased until a criterion for detection is reached; again strength is decreased and so forth. Figure 4 illustrates this procedure. Stebbins (*13*) has modified the tracking method and applied it in his extensive research in studies of ototoxicity. It has also proven effective in assessing the effects of neural deficit on visual function in cats (*14*).

An Alternative Psychophysical Approach

Although operant methods can be modified to provide efficient posttreatment data, the need for extensive pretraining can be a serious drawback. For example, when equipment and manpower are limited, operant methods are more appropriate for research requiring a small number of subjects. In toxicological research, where irreversible treatments are combined with a desire to describe a complete dose-response relationship, it is often desirable to use a large number of subjects and thus more efficient pretreatment procedures.

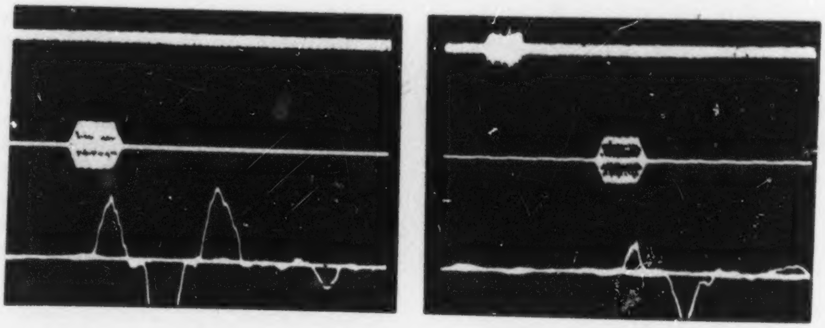

FIGURE 5. Oscillographic records of startle trials (left) without and (right) with a noise burst stimulus preceding a startle tone; (upper trace) background noice and noise prestimulus; (middle trace) 10 kHz startle tone; (bottom trace) startle response.

Researchers at several institutions, including Johns Hopkins University, have been working on the development of a psychophysical procedure that promises to be simple and quick to implement and which may prove nearly as sensitive as procedures based on extended operant training. This method is based on the inhibition of the acoustically elicited startle reflex by a prior stimulus.

Several authors have described the phenomenon known as prestimulus inhibition (*15*). In essence, low intensity stimuli, when presented 20 to 200 msec before the startle-eliciting stimulus, reduce the amplitude of the startle response. Figure 5 illustrates the phenomenon. The prestimulus may be auditory (*16, 17*), visual (*18–20*) or cutaneous (*21, 22*). The prestimulus need not be the onset of a stimulus; termination of an ongoing stimulus (*23*) or changes in the frequency distribution of an ongoing noise (*24*) will also inhibit startle. Startle amplitude reduction appears to be maximal when the prestimulus and startle stimulus are separated by 40–80 msec; the particular maximum seems to vary slightly depending on the nature of the prestimulus Similar inhibitory effects of preceding stimuli are also seen with other reflexes, such as the airpuff- or tap-elicited eyeblink.

From the perspective of the animal psychophysicist, the reflex inhibition finding is of interest because the amount of inhibition varies with the strength of the prestimulus. Further, at least in audition the intensity of the prestimulus just required for inhibition is quite close to the absolute threshold of detection (*25, 26*). This observation suggests that the reflex inhibition technique could be used as an animal psychophysical procedure. Since it is based on a reflex rather than a learned response, the training procedure could be eliminated and the pretreatment period considerably shortened. Thus, large numbers of subjects could be tested relatively quickly. Virtually all mammals seem to show the startle reflex and may therefore be used as subjects. While birds do not exhibit auditory startle, they have a visual startle response which is similarly modulated by prestimulus presentations. Indeed, the stimulus parameters effective for modulation of the two reflexes seem nearly identical (*27*).

Although this is a new method, a recent study by Russo (*22*) has applied it to sensory system toxicity. In his research, which examined auditory and cutaneous sensitivity, Russo employed pairs of startle trials. Each pair compared the elicited startle with and without a prestimulus. The criterion of inhibition was that, within each pair of trials, the trial with a prestimulus produced a smaller amplitude response than the trial without the prestimulus. Following each pair of trials, Russo manually adjusted stimulus intensity to track the threshold of startle inhibition. Thresholds for 1/3-octave noisebands and electrocutaneous stimuli were determined in this fashion and were similar to those obtained in operant discrimination studies. Further, the method detected temporary auditory threshold shifts induced by extended exposure to noise and shifts in electrocutaneous thresholds induced by Lidocaine injections.

In preliminary experiments at Johns Hopkins, we are working on a modification of Russo's procedure, which we hope to apply to assessment of visual function. In particular, we are developing a system which relies upon the graded nature of the startle response, rather than an all-or-none criterion of inhibition. This procedure yields psychometric functions that relate the amplitude of the startle response to the parameters of the prestimulus.

Figure 6 shows data from a single rat for a single 72-trial session based on this procedure. For this pilot work the prestimulus was white noise. Vary-

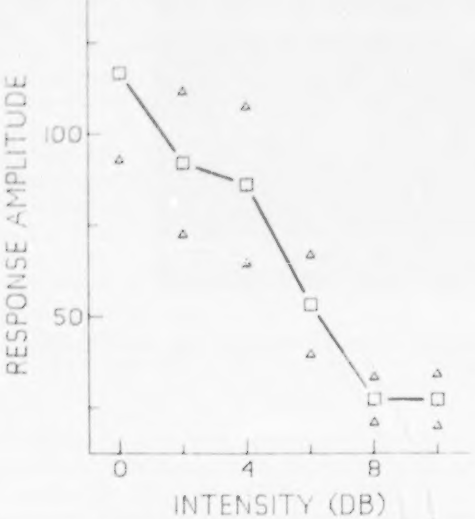

FIGURE 6. Sample data from a reflex-inhibition technique. Amplitude of an elicited startle response was measured as a function of the intensity of an auditory prestimulus. The inverse relation between response amplitude and stimulus intensity reflects the inhibitory effect of the stimulus. Each data point (square) is based on 12 trials during the subject's first exposure to the testing procedure. The small triangles show the standard error of the mean, which decreased by 50% on the following test day.

ing intensities were presented along with control trials in random order (method or constant stimuli). An advantage of the constant stimulus method is that several subjects may be run at the same time, with only a single stimulus-generating apparatus. We think these data are quite acceptable for such a brief run; sessions can be much longer, given appropriate intertrial intervals to preclude the habituation of startle.

Over the past several months, we have been developing an automated test apparatus which will enable us to test up to four subjects at once. We are beginning studies of normal subjects, using a variety of visual tasks as well as pure-tone audiometry and electrocutaneous sensitivity. These data will serve as baselines for a series of positive control tests with toxins known to affect these systems (aminoglycosine antibiotics, MeHg, CS_2).

The reflex inhibition procedure does have several limitations as a psychophysical technique. Its principal constraint is that it has a limited dynamic range, since stimuli of low intensity will produce large decrements of startle amplitude. Further, the method requires extreme care in the control of the testing environment. Extraneous stimuli, even in modalities other than that under study, can inhibit the reflex and make it impossible to partial out the effects of the stimulus under investigation. Lastly, the procedure is necessarily limited to detection studies, as any detected stimulus will alter the reflex. This limitation contrasts with the broader range of discrimination that may be studied with operant or classical conditioning techniques.

Despite its limitations, the reflex inhibition procedure promises to be a psychophysical technique of great utility for the toxicologist, who frequently wishes to test large numbers of subjects. At the present time, it may represent an optimal combination of speed and sensitivity.

Figures 1-4 and 6 were prepared by a computer graphing program (*28*).

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Environmental Health Perspectives
Vol. 44, pp. 55-62, 1982

Newer Laboratory Approaches for Assessing Visual Dysfunction

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The crucial point that will be emphasized throughout this report is the potential utility of analyzing visual cortical receptive field (RF) properties at the single-cell level as a sensitive and reliable neurotoxicity screening tool. Numerous studies employing exposure of kittens to altered visual environments during the critical period have demonstrated that particular classes of RFs can be selectively affected while sparing others. There has been a rapid proliferation of new methods used to investigate such effects. An important current trend involves the development of multidisciplinary combinations of approaches. The various maneuvers reviewed here seem adaptable to studying neurotoxic insult of the sensitive properties of cortical visual neurons, particularly in the cat or monkey. Conceivably, a general disruption of cortical RF properties might be expected following toxic exposure since individual RF properties are generally not determined by completely independent mechanisms. In fact, some toxicants might produce a general degradation of RF properties akin to the electrophysiological results reported for long-term dark rearing or binocular deprivation.

Visual Cortical Receptive Fields and Their Properties

Over the past 20 years, a number of basic concepts have emerged concerning the organization of the mammalian visual system, particularly at the cellular level. A centrally important concept, which ultimately describes the fundamental building blocks of visual circuitry, is the receptive field (RF). Each neuron along the retino-geniculo-cortical pathway (as well as other visual pathways) is concerned with the analysis of a small subregion of the total visual field. At each level, the RFs are arranged in a systematic topographic (i.e., retinotopic) manner such that the entire visual world is laid out multiply across the occipital cortex with an overrepresentation of the central region. A specific stimulus presented within the boundaries of a cell's RF will evoke a maximal discharge of impulses from that cell. The particular stimulus pattern evoking the strongest response is said to embody the "stimulus trigger features" or RF properties of that cell. For example, a well-known stimulus requirement of cortical units is orientation or angular position of the "best" or optimal stimulus.

Although there is a significant literature on primates, most of our knowledge of how RF properties code form, movement, and depth in the visual world has been obtained from the feline visual system by using recently developed microelectrode recording and stimulating techniques. The domestic cat has been so widely used as a subject that its use in the vision laboratory has been characterized as ". . . currently representing the primary source of neural building blocks from which the majority of models of human vision are constructed . . . " (*1*). In a series of pioneering studies on the feline visual system, Hubel and Wiesel (*2, 3*) laid down the basic foundation for interpreting the specific RF properties of cortical visual neurons.

The two RF properties that have received the most attention are ocular dominance and orientation selectivity. In the normal mammalian visual cortex, the majority (80-85%) of cortical neurons

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receive excitatory input from each eye, i.e., they are binocular. The remaining small population of cells, categorized as monocular units, receive input from one eye or the other. Furthermore, for cells receiving a dual input, that input is not necessarily equal for the two eyes. A seven-class scheme or an ocular dominance (OD) index characterizing this relative ocular input to a particular cortical cell was proposed by Hubel and Wiesel (*2*). This nomenclature has been universally adopted among visual neurophysiologists, and probably represents the easiest RF property to determine. A typical OD histogram and definitions for the various categories appear in Figure 1. For a normal adult cat, the distribution for a population of cortical cells is bell-shaped with some contralateral skewing.

Of all the specific stimulus trigger features of visual cortical RFs, perhaps the most fundamental requirement is orientation specificity. That is, the appropriate stimulus must assume some critical angular position within the boundary of the RF for maximal stimulation of that cortical unit, and any deviation (± 10-25°) from the optimal orientation results in dramatically decreasing or eliminating the neural response. Typically, angular selectivity is determined by moving an appropriate stimulus, usually a light or dark edge (e.g., a bar), through the RF at different orientations while measuring the response. The response profile of a single cortical cell as a function of stimulus orientation is referred to as the orientation tuning curve (OTC), depicted in Figure 2 for an hypothesized RF. An OTC can be compiled by one of two RF plotting techniques. The first consists of simply listening to the amplified response of the unit over an audio monitor while simultaneously monitoring the activity on an oscilloscope (the manual/auditory feedback method popularized by Hubel and Wiesel) (*2*, *3*). The second approach, one advantage of which is to eliminate possible experimental bias, uses a computer or automatically controlled visual stimulation system. Measurements of orientation preference and the degree of selectivity made by these two methods generally stand in good agreement with each other (*4*).

There exists an elegant layout of ocular dominance and orientation specificity within the cortex which has been convincingly demonstrated electrophysiologically and anatomically in both the cat and monkey striate cortex (*5*-*8*). Two independent sets of vertical "columns" or cortical slabs, ocular dominance and orientation, represent the fundamental building blocks of visual cytoarchitectonics. Cells favoring the same eye or preferring approximately the same optimal stimulus orientation are grouped into functional slabs arranged perpendicularly to each other and the cortical surface and through the six layers of gray matter reveal systematic changes in both ocular dominance and orientation preference. Orientation changes occur in a clockwise or counterclockwise direction such that 180° is represented across about 1 mm of cortex before a reversal. All orientations are about equally repre-

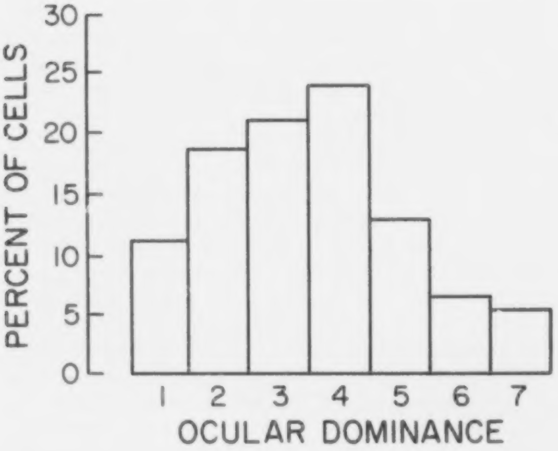

FIGURE 1. Ocular dominance histogram showing the relative influence of the two eyes on a sample of cortical units in a normal adult cat. Each cell is assigned to one of seven categories: Monocular cells are driven exclusively by the contralateral eye (1) or the ipsilateral eye (7), while binocular cells are driven equally by each eye (4), more strongly by the contralateral eye (2 or 3), or more strongly by the ipsilateral eye (5, 6).

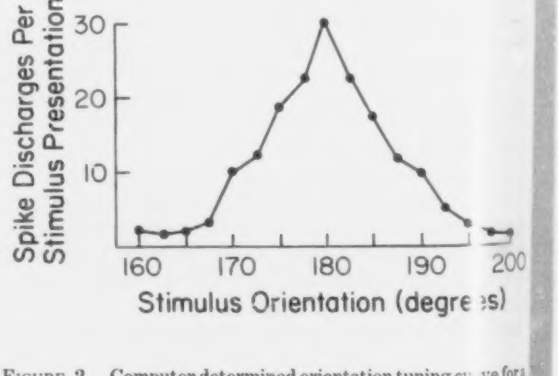

FIGURE 2. Computer determined orientation tuning curve for a cortical cell's receptive field, showing the response to a slit of light at several orientations. This cell is tightly tuned for a stimulus oriented near 180°.

sented with neighboring columns varying by about 10-15°. Likewise, ocular dominance changes progressively; one eye is dominant, then the two become roughly equal, and finally the other eye becomes dominant in roughly the same cortical distance but independently of a 180° orientation sequence. One complete set of these columns, an orientation cycle or ocular dominance cycle, has been termed a hypercolumn.

Another RF feature characteristic of most normal cortical neurons is that a more vigorous response is elicited by a moving than a stationary stimulus. Furthermore, the majority of cortical units display a directional preference, responding more strongly to movement of the optimal stimulus in one direction than in the other direction. This is illustrated in Figure 3; the cell prefers the appropriate stimulus moving in a downward direction orthogonal to the optimal orientation. Other RF properties of cortical visual neurons include the spatial layout of excitatory and inhibitory subregions (shape) which compose a RF, preference for specific velocities of a moving stimulus, and interocular relationships such as RF locational or orientational disparities of binocular cells.

Based on the uniformity in RF properties, cortical units have been categorized by various investigators. The most commonly used system comes from the early studies of Hubel and Wiesel (*2*, *3*) who proposed the now classic simple, complex, and hypercomplex RF types. Extraction of stimulus features from the visual world, as conceived by these authors, is carried out by a hierarchical arrangement of these three basic RF types in visual cortex. In this model the properties of simple cells' RFs are determined by direct excitatory input from a number of lateral geniculate cells, while the RFs of complex cells are determined by input from several simple cells. Complex cells, in turn, provide the input to the RFs of hypercomplex cells. This strictly serial model, however, has been seriously challenged on both anatomical and electrophysiological grounds (*9*). The central visual circuitry, particularly from retina to cortex via the dorsal lateral geniculate nucleus, comprises several functionally distinct, parallel-running subgroups of cells, two of which have been called the *X*- and *Y*-pathways. At the cortex, these separate systems are less easily identified, although several electrophysiological studies point to a strong correlation between particular RF types and these parallel afferents. It is currently thought that the *X*-system influences mainly simple cells while the *Y*-system influences mainly complex cells. Important clues to understanding the functional roles of the *X*/*Y* dichotomy have been provided by differences in retinal distribution, spatial and temporal stimulus properties defined electrophysiologically, and cortical synaptology. The *X*-system is primarily concerned with detailed, central vision and binocular stereoscopic vision, while the *Y*-system subserves some functions of peripheral vision, such as the detection of motion, location, and possibly coarse outlines.

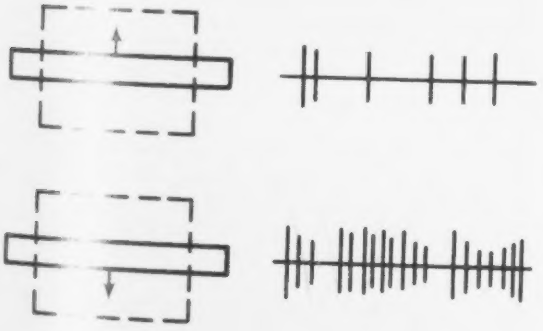

FIGURE 3. Schematic representation of directional selectivity for the same unit as in Fig. 2. Shown on the left is a slit of light moving through the receptive field in both directions, orthogonal to the optimal orientation. On the right are responses evoked by these stimuli.

Neuroelectric Approaches

Generally, more varied and advanced methods of assessment exist for the visual system than for other sensory systems. Like all sensory systems, its development is susceptible to environmental factors, including chemical agents; i.e., although genetics defines the potential, a high level of neuronal plasticity is present. Postnatal plasticity is greatest during early development, a time referred to as the critical period (CP), since normal visual experience during this time is crucial to normal development: Absences or abnormalities in visual experience are manifested as long-lasting functional and physiological deficits. For example, occluding one eye (monocular deprivation or MD) of a kitten during the traditionally defined CP, 1-4 months of age, results in a loss of vision through that eye and a severe shift in ocular dominance in favor of the experienced eye (*10*). It is of particular interest to visual scientists to determine the extent to which RF properties as well as overall visual physiology are influenced by innate factors, passive maturational changes, and environmental contributions. That is, at each developmental stage, to what extent is the system susceptible to impairment or loss of function due to various degrees of abnormal experience?

Since alterations in neural function following exposure to toxic substances, especially at long-term low-level exposures, are often quite subtle, and because it is necessary to determine threshold levels for toxins, it is desirable to have measures of nervous system function which are very sensitive. One suitable tool may be the neurophysiological method of single-cell recordings. RF properties of single cells are finely tuned, and are highly sensitive and responsive to environmental manipulations during the CP. In particular, the properties of ocular dominance, orientation preference, and directional preference have demonstrated highly robust effects. For example, a number of innovative studies (*11*, *12*) have found that if only one orientation (e.g., vertical) is experienced during the CP, most cortical cells will be tuned to that orientation.

In most of these "environmental surgery" studies, animals have been exposed to a unique visual experience throughout the CP. However, some researchers, with the goal of determining the sensitivity of the system, have attempted to delineate the minimal amount of experience needed to alter RF properties. One interesting, but controversial, study (*13*) reported that as little as 1 hr of exposure to a highly redundant environment (vertical gratings) could significantly alter the normal orientation distribution in kitten cortex, i.e., disproportionately many cortical units were tuned to vertical orientations. Others have shown that ocular dominance can be dramatically shifted following only a few hours of monocular exposure during the peak of the CP (*14*). On the other hand, important differences in the degree of susceptibility of certain RF properties to environmental insult are beginning to be unraveled. Velocity preferences, for example, appear to be more resistant to a "matching effect" than any other RF property (*15*). Others contend that certain subclasses of orientation preference are also quite resistant to environmental manipulations, pointing to a strong genetic determination (*16*). In short, this sort of selective versus instructive question currently represents a major controversy among visual neurophysiologists. Since exposure to toxic substances can be restricted to either prenatal or postnatal periods, or be of a more chronic nature throughout adult life, knowledge of such developmental differences and their chronologies provides still another means for measuring the relative sensitivity of the visual system to neurotoxicity at the level of individual RF properties.

The effect of chemical agents on RF properties is a topic which has scarcely been broached, but indications are that this might be a highly sensitive and responsive measure, useful for revealing mechanisms of dysfunction. For instance, two observations made on cats during acute single unit preparations are: administration of barbiturate anesthetic agents leads to a sharpening of orientation tuning of visual cortical units (*17*) and improper regulation of oxygen, as measured by end-tidal CO_2 concentration, leads to a rapid and severe degradation of visual RF quality, e.g., high erratic base rate and poor responsiveness (*18*). Another study, explicitly addressing changes in visual RF properties following neurotoxic exposure, reported that the widely used organophosphate pesticide, mevinphos, and related cholinergic drugs (agonists such as pilocarpine or antagonists such as atropine sulfate) abolish the highly tuned directional selectivity of single cells in the avian nucleus rotundus (*19*). A similar effect was reported following ethanol exposure. These effects may be the neuronal basis for visual behavioral impairment at certain levels of intoxication (decrease or loss of visual attention, orientation behavior, and peripheral vision).

Another neuroelectric technique, one which has already been widely adapted for neurotoxin testing, is the visually evoked potential (VEP) (*20*). The VEP is a gross recording, obtained through scalp or depth electrodes, which simultaneously measures thousands of cells' responses to visual stimulation. The EP signal is generally localized to the small area of brain directly below the electrodes. Since the response is obscured in a single trace due to trial-to-trial variability, inherent noise, and background EEG, a reliable EP record is obtainable only through signal averaging techniques. A standard cortical VEP (VECP) record evoked from visual cortex by a light flash is diagrammed in Figure 4. Like all EPs, the VECP is a complex waveform consisting of a series of positive (P1, P2, P3) and negative (N1, N2, N3) peaks, the amplitude and latency of which are determined by a number of factors: stimulus parameters, location and mode of recording and age and physiology of the animal. Two phases comprise the VEP, and

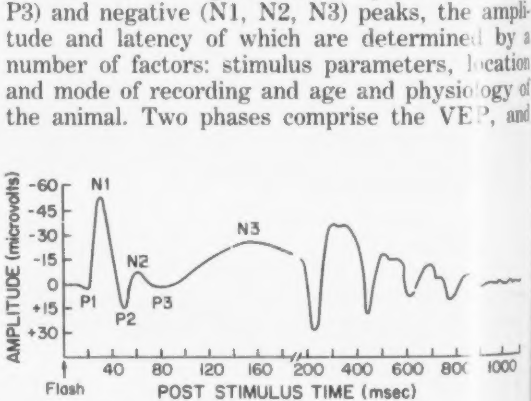

FIGURE 4. Electrical activity in rat visual cortex following a light flash, showing the various components of the evoked potential and the subsequent 3-6 Hz afterdischarge. After Fig. 1 of Dyer et al. (*20*).

alterations in peak onset latency or amplitude (baseline-to-peak or peak-to-peak) of specific components may reveal CNS dysfunction. The early primary components (latency < 50 msec), P1-N1 and N1-P2 are most sensitive to changed input parameters and are commonly thought to depend upon retino-geniculate activity, while the late secondary components (50-200 msec latency), P2-N2, N2-P3 and P3-N3, which are believed related to diffuse activity of thalamic, mesencephalic, and cortical origins, are quite variable in waking brains and are suppressed by anesthetics.

This technique has several advantages over single-unit records: (1) it can be quickly, easily, and repeatedly applied, and (2) it can be applied to humans as well as animal subjects. Its use as a research tool for determining mechanism of dysfunction, however, is restricted to a molar level. Clearly, it is of considerable theoretical and practical importance to establish correlations between specific parameters of gross evoked potentials, on the one hand, and the activity of various classes of single units on the other hand. A number of studies have approached this question (*21*), but a comprehensive picture has not yet emerged.

The ease with which the VEP can be measured has made it a prime candidate for clinical use, epidemiological or experimental field studies, and longitudinal studies. It is currently used as a diagnostic tool for amblyopia in very young children and other nonverbal patients. Also, it has been proposed for use in evaluating neurotoxicity in individuals who have had prolonged occupational contact with possibly toxic substances. However, little toxicological information is actually obtained from human subjects since controlled administration of toxins cannot exceed threshold levels. For developmental or longitudinal studies, the VEP is well-suited since components emerge in a chronologically orderly sequence and the waveform, which does not assume adult characteristics until a relatively late developmental stage, changes again during senescence (*22*).

The VEP is currently being used to study effects of such common toxicants as CO, some heavy metals (lead, methylmercury), and some organophosphate compounds (mevinphos). For example, one study of the effects of methylmercury (*23*) reported that rats prenatally exposed to this heavy metal (their mothers ingested a single low dose) showed visual dysfunction, as measured by the VEP, in adulthood. The early primary VECP components in these animals had an increased amplitude relative to controls, indicating a disruption of function in the retinogeniculate-striate system. Also, the P2 and N2 peaks had a shortened latency, which in conjunction with other reports, suggests a greater susceptibility to damage by methylmercury for slowly conducting axons than for other axons.

A common feature of mammalian visually evoked cortical activity which follows the VEP (120-200 msec latency) and persists for 2 sec or longer is a rhythmic 3-6 Hz repetitive discharge known as the visually evoked response afterdischarge (VER AD) (Figure 3). It is generated and maintained by recurrent circuitry at the dorsal LGN level and is modulated by a variety of limbic, brain stem, and thalamic systems (*24*, *25*). This response, which has only recently been explored, is a sensitive measure of visual dysfunction caused by some substances when the VEP is insensitive. The VER AD is especially sensitive to drugs such as convulsants and anticonvulsants having a predominantly thalamic level of action. Thus one promising use of the VER AD is as a model for the treatment of epilepsy.

Neurochemical Approaches

Studies of chemically induced changes in single visual cortical neurons are quite limited, although the development of new neurochemical techniques has made this a major objective. Potentially fruitful approaches for assessing the effects of both acute and chronic administration of toxic substances to developing and adult visual cortical neurons have been provided by studies of specific functions of neurotransmitters in visual cortex. The value of this technology is that it permits relatively specific pharmacological control of locally applied chemicals to sensory regions (e.g., visual cortex) of the brain and concurrent or subsequent electrophysiological evaluation of specific, patterned cellular activity evoked by appropriate sensory stimuli. In particular, two techniques have been used in elucidating neurochemical mechanisms operating in the visual cortex of the cat. Importantly, both allow for within-animal controls, such as interhemispheric comparisons.

The first preparation, most suited to ascertain immediate or acute effects of exposure to toxic substances, is the microiontophoretic administration of substances of interest while simultaneously recording single-cell activity. Briefly, this technique involves penetrating the visual cortex with multibarrel micropipets. Typically, the center barrel is used as a recording/marking electrode for single units while the other barrels contain agents to be released at specific sites by passing a small ejecting current through the desired solution. For example, Sillito (*26*) and others (*27*) have demonstrated in visual cortex that the action of γ-aminobutyric acid (GABA), an important inhibitory neurotransmitter in the mammalian brain, can be reversibly antagonized by iontophoretic application of the alkaloid, bicuculline. The general effect of bicuculline on single cells is a disruption of response specificity for stimulus parameters such as orientation and direction of movement. In addition to the general disruption of RF properties, specific differences were noted for RF types such as simple versus complex. A corollary question, arising from these specific cortical effects following neurochemical manipulations, is: To what extent are specific differences manifested systematically throughout the central visual pathways following environmental insult, such as from toxic exposure, since functionally different systems (e.g., the *X*/*Y* dichotomy) are believed to be connected to these different cell types? Such an assessment would offer a means for measuring the relative sensitivity of the visual system to neurotoxicity at a more integrative level. The primary impetus for investigating these functional differences has already been provided by studies which have demonstrated that special critical period exposure for kittens can selectively disrupt normal function of the *Y*-system, while the same maneuver, in this case paralysis of one eye, performed on the adult cat degrades the *X*-system (*28*).

Equally important questions concern changes in visual cortical RF properties following chronic administration of chemical agents, or low-level exposures during early postnatal development. Consequences in the latter case may not be fully manifested until late in life and may thus demand longitudinal analysis. A preparation, quite adaptable for these maneuvers, involves a continuous microperfusion technique using the newly developed Alzet osmotic minipump. Like the first technique, it, too, has yielded a means for coupling neurochemical and electrophysiological approaches in the study of single neurons in the developing and adult visual cortices. This simple procedure involves subcutaneously implanting a minipump system with a connecting cannula that delivers the contents slowly and continously over a specific cortical site. After the contents have been emptied, minipump replacement, if so desired, can be performed easily and quickly. Subsequently, electrophysiological examination of single neurons is performed, using conventional procedures. Most recently, this preparation has been employed to investigate neurochemical mechanisms that possibly control the time course of the postnatal CP as manifested by the monocular deprivation effect in the feline visual system. Intraventricular injections (*29*) or local cortical microperfusions via an implanted osmotic minipump/cannula system (*30*) of the neurotoxin, 6-hydroxydopamine (6-OHDA), prevent the loss of binocularity which normally follows monocular deprivation during the CP. These findings, along with subsequent studies, indicate that a normal balance of catecholamines plays a crucial role in promoting neuronal plasticity in the feline visual system. For example, concurrent microperfusion of the visual cortex with 1-norepinephrine HCl blocked the 6-OHDA effect for monocularly deprived kittens, who then showed the expected shift in ocular dominance (*31*). Furthermore, the susceptibility to monocular deprivation of kittens well beyond the CP (13 weeks) and, to a lesser degree, adult cats (2 years old) is restored by extended periods (2 weeks) of cortical microperfusion with norepinephrine (*30*).

Behavioral Approaches

Behavior is the most complex but also the most relevant level at which the effects of environmental manipulations can be examined. The assessment of visual behaviors, which has been extensive in the cat, has been approached in various ways. The first has involved observing and cataloguing naturally occurring visual behaviors in the normal adult organism. A second approach, related to the first, entails evaluating the development of such behaviors and various factors that affect them, especially during development. Commonly studied visual behaviors have traditionally included gross visuo-motor responses such as the looming reflex, visual orienting, following and pursuit of visual targets, obstacle avoidance in an open field, triggered visual placing, coordinated jumping, and depth perception as tested on a visual cliff. Unfortunately, a good correspondence between visual deficits defined physiologically at the single-cell level and behaviorally via gross visuo-motor performance may not occur. These behavioral tests are often not sensitive enough, and further, extensive visuo-motor adaptation can mask real deficits or anomalies of visual function. Tests using refined and controlled psychophysical procedures have provided the necessary sensitive measures of visual function in normal and experimental animals. Attention will be confined to these procedures since they offer a method for pinpointing the nature and chronology of sensory deficits and, more importantly, a means for comparison of human and infrahuman results.

Two behavioral tests, a nose-key operant (*4*, *3*
and a modified Lashley jumping stand (*33*) require an animal to perform in a two-response forced-choice discrimination task. These tests have con

siderable appeal since training is relatively fast and easy, allowing a variety of visual functions with known parameters for humans to be determined: acuity, orientation perception, depth perception, vernier alignment, and contrast sensitivity. With these techniques the functional significance of single-unit disruption can be directly addressed; i.e., does the quality of RF properties define visual capacities? Several studies have provided partial answers. A kitten monocularly deprived during the critical period has a mostly monocular cortex, as seen by single-unit studies (*10*). The behavioral manifestation, determined using a conditioned suppression paradigm, is that binocular visual functions (e.g., stereopsis) are absent or extremely attenuated (*34*). A second manipulation, which alters the cortical distribution of RF orientations, has been shown to affect perceptual performance as well. Kittens reared experiencing a restricted range of orientations (horizontal or vertical) develop biased visual cortices (i.e., most units are tuned to horizontal and vertical, respectively) and are visually more sensitive to the experienced orientations (*4*).

Conclusions

The relative sensitivity of visual cortical RF properties to environmental influences suggests a potential utility of RF analysis as a neurotoxicity screening tool. New and improved laboratory techniques including neuroelectric, neurochemical, and behavioral approaches adaptable to this area of research were briefly reviewed. Their usefulness as screening tools remains to be fully demonstrated.

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Environmental Health Perspectives
Vol. 44, pp. 63-66, 1982

Sensitive Periods of Susceptibility to Auditory Trauma in Mammals

by James C. Saunders* and Chia-Shong Chen*†

Evidence is presented to support the hypothesis that the cochleae of young animals are more susceptible to auditory trauma than the cochleae of the adult. A sensitive period of heightened susceptibility to acoustic trauma from noise exposure has been demonstrated in three mammalian species. The cochlear pathology associated with this trauma is severe damage to the outer hair cell system. Abnormal growth of auditory evoked responses recorded in central auditory nuclei accompanies the receptor damage during the sensitive period. There is evidence of a similar sensitive period of susceptibility to cochlear insult from ototoxic drugs. The time frame of the sensitive period may be different for drug or noise insult to the cochlea, but the principal pathology of outer hair cell loss remains the same in both cases. The implication of these sensitive periods to auditory trauma, for human development is considered.

Introduction

The question of whether the developing ear is more vulnerable to auditory trauma than the adult ear has a short history. However, animal models of early auditory trauma during the past 5 to 7 years offered some interesting observations, and it is the purpose of this paper to summarize some of these findings.

Is the Developing Ear More Susceptible to Acoustic Trauma?

Data from five mammalian species support the hypothesis that exposure to intense sound in the developing ear is more traumatic than in the adult ear. We will examine these data with respect to exposures that produce either a permanent hearing loss or temporary hearing loss. We will also consider data which indicate that the cochlea passes through a sensitive period of enhanced susceptibility to acoustic trauma. The central consequences of this trauma will also be noted.

Permanent Damage

The cochlear microphonic (CM) response has been measured in 8-week-old kittens and adult cats (*1*) 30 days after exposure to intense pure tones (50 min of a 5.0 kHz tone at a sound pressure level (SPL) that varied somewhat between test groups, but which was typically 105 dB). The results, for certain stimulus levels, indicated a loss in CM sensitivity in the kittens which was greater than in the adult. The CM response reflects the integrity of the cochlear receptor cells (particularly the outer hair cells) and it is likely that the kitten hair cells were more traumatized by the exposure than those in the adult. Noise exposure in the neonatal guinea pig (30 hr of white noise at 120 dB SPL) beginning at either 2 or 8 days postpartum has also been shown to produce significantly greater cochlear pathology (loss of outer hair cells) than the same exposure in the 8-month-old adult (*2*). This observation has been extended to less traumatic noise exposures in neonatal and adult guinea pigs (*3*). It has also been shown that the auditory system of the guinea pig fetus in utero is susceptible to noise exposure (*4*). In the guinea pig studies, the pathology observed along the neonatal organ of Corti was generally the same. Hair cells were permanently damaged, and in particular the outer cochlear hair cells suffered the greatest loss. The inner hair cells were rela-

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tively unaffected. This evidence indicates that the cochlea of younger animals is more severely traumatized by intense sound than that of the adult.

Temporary Fatigue

The permanent loss of cochlear structures and function cited above may represent one aspect of a more general process within the receptor organ. If younger ears are more generally susceptible to overstimulation, then exposure to sound at levels that do not cause permanent hearing loss, ought to produce heightened levels of temporary auditory impairment. The process of auditory fatigue was examined in five groups of neonatal hamsters at selected ages between 15 and 85 days. The animals were anesthetized, a recording electrode was placed in the inferior colliculus, and the ear was exposed to intense pure tones (10 min of a 3.0 kHz tone at 110 dB SPL) (*5*). Inferior colliculus evoked responses were used to test auditory threshold sensitivity. Threshold shift (TS) 1 min post exposure was lowest in the 15- and 85-day-old groups, and showed complete recovery to the pre-exposure threshold within 100 min. At 40 days, however, the threshold shift was greatest, and recovery took much longer than 100 min. These data are important, for they indicate that the young cochlea is sensitive to overstimulation at both high and moderate levels. Moreover, the results also show a period of enhanced susceptibility to auditory fatigue which reached a peak around 40 days of age.

Sensitive Periods

The observation of a heightened period of susceptibility to acoustic trauma as demonstrated in the hamster, has also been shown in other experiments. These studies, conducted with mice, hamsters and rats, systematically varied the neonatal age at which the animals were exposed. The recovery interval (between 5 and 14 days), as well as all other test conditions were held constant for each group. It has long been known that certain strains of mice can be rendered susceptible to audiogenic seizures by exposure to sound during a specifically defined time after birth (*6*). The noise exposure that renders these mice susceptible to seizures is remarkably mild (90-120 sec of wide or narrow band noise at 100-110 dB SPL) given the profound effect it has on the animal (*6*). Recent work investigating the physiological basis of the sensitive period has revealed that the cochlea is severely damaged by noise exposure at this time (*7*). Furthermore, the senstivity of CM-isopotential thresholds or cochlear nucleus evoked-response thresholds may show a loss of between 25 and 40 dB after noise exposure during the sensitive period (*8*, *9*). Exposure to the same noise in older animals (40-50 days) has relatively little effect on threshold sensitivity (*9*). The most sensitive period in the C57BL/6J mouse appears to be between 17 and 19 days while in the BALB/c mouse it occurs between 21 and 23 days. Similar studies have revealed a sensitive period for acoustic trauma in the hamster between 30 and 40 days (*10*, *11*) and in the rat between 20 and 25 days (*12*). Again, the same exposure conditions presented to hamsters older than 65 days or rats older than 40 days has no traumatic effect. The characteristic time course of the sensitive period for acoustic trauma in these three species is summarized in Figure 1. In this figure, the percent of maximum threshold loss at the most affected frequency is plotted as a function of age.

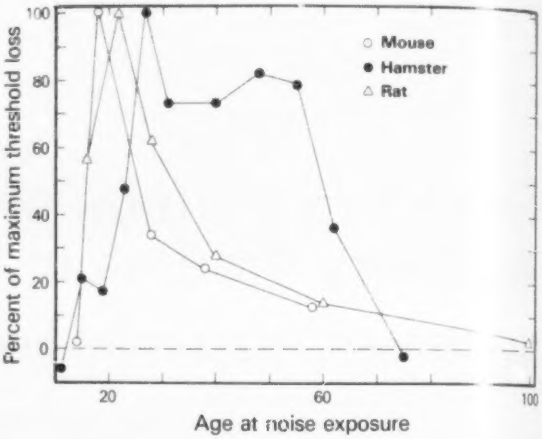

FIGURE 1. Time course of the sensitive period is plotted in three species: (○) mouse; (●) hamster; (Δ) rat. The curves represent the percent of maximum threshold loss at the most affected frequency across time. Redrawn from Saunders and Tilney (*13*).

In mouse, rat and hamster, the onset of the sensitive period corresponds well with the final stages of functional and structural maturation of the cochlea. The cochlear histopathology observed in both rat and mouse after noise exposure during the sensitive period is geneally the same. There is massive outer hair cell damage widely distributed throughout the cochlea (*7*, *14*, *15*). Remarkably, the inner hair cell system appears undamaged. These observations are similar to those reported in the kitten and guinea pig; however, the degree of outer hair cell loss in these species was less pronounced than that reported for the mouse and rat. In the kitten and guinea pig, the age at exposure was not manipulated specifically, and the results in these neonates may lie outside the most effective time of the sensitive period.

Central Consequences

In two of the species studied (hamster and mouse) an interesting phenomenon in the central auditory pathway accompanies the loss in peripheral auditory function after noise exposure during the sensitive period. There is evidence from a number of sources that outer hair cell pathology is associated with the abnormal growth of intensity in central auditory nuclei, or in the animal's behavioral judgement of loudness, with increasing stimulus intensity. Abnormal growth in evoked response amplitude with increasing stimulus intensity has been observed in the inferior colliculus and the cochlear nucleus of the mouse and hamster (*16-18*). The interesting feature of this abnormal growth in response amplitude in the noise exposed animals is that at high stimulus levels, the response far exceeds that seen in control animals. For example, in the BALB/c mouse exposed to noise at 21 days of age and then tested at 27 days of age, the cochlear nucleus evoked response to a click stimulus at 95 dB SPL averages about 220 μV. Control animals at the same age show a similar evoked response amplitude to a 95 dB click. At 110 dB SPL, however, the noise-treated subjects exhibit a 510 μV response, whereas the control animals show only a 305 μV response! The abnormally large response to intense sound in the noise exposed neonate has been called over-recruitment (*16*) and an example of the growth in evoked-response amplitude with increasing stimulus intensity, in control and noise-exposed mice, is presented in Figure 2.

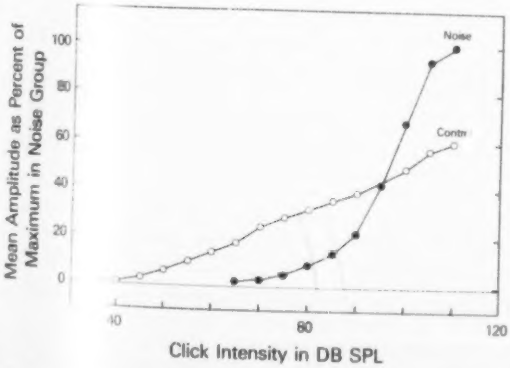

FIGURE 2. Growth in evoked response amplitude for a group of noise-exposed and control BALB/c mice. The noise exposure occurred at the peak of the sensitive period in this species. Amplitude is plotted as a percent of the maximum response in the noise group. Redrawn from Saunders and Bock (*18*).

Recent evidence also shows sustained after-discharges in the post-stimulus time histograms of inferior colliculus cells recorded in noise exposed neonatal mice (*19*). Similar after-discharges are not seen in the inferior colliculus cells of control mice. Whether or not this is the cellular counterpart of the "over-recruitment phenomenon" has yet to be determined. Furthermore, the peripheral (cochlear) or central origins of over-recruitment are not yet understood.

Is the Developing Ear More Susceptible to Ototoxic Trauma?

There is recent evidence suggesting a sensitive period for ototoxic insult to the cochlea. Mice of the BALB/c strain have been exposed to the ototoxic amino glycoside, kanamycin, and the data suggest that a 16-day treatment period (one injection of 400 mg/g per day) between 5 and 21 days produces a 40% to 60% loss of outer hair cells in the apical turn and a 70% to 90% loss in the basal cochlear turn. A 10-day treatment from 17 to 27 days produces a 40% loss of outer hair cells throughout the cochlea (*7*). In both instances the inner hair cells appeared normal. Unfortunately age and number of injections were confounded in this study, making it difficult to conclude if the pathology was due to age or number of injections. However, results from a recent experiment using the acoustic priming model to evaluate the ototoxic effect of kanamycin suggest that BALB/c neonates (10 to 14 days old) are at the peak of the sensitive period to ototoxicity, (Chen, personal communication). The dose levels used in this study were also 400 mg/g per day, but the number of injections was held constant for all age groups. The utility of the acoustic priming model for assessing the effects of ototoxic drugs has been discussed elsewhere (*20*). It is interesting to note that a 16-day injection period in the adult mouse (C3H strain) had a far less traumatic effect on hair cells or hearing (*21*). Data for the rat pup also indicate a profound cochlear pathology of outer hair cells, and a loss in auditory function, accompanying kanamycin treatment during days 11 to 20 postpartum. Identical treatments from the days 1 to 10 or from day 15 to day 23 had relatively little effect on auditory function or on cochlear structures (*22*). The profound loss of outer hair cells in these ototoxic poisioning experiments was the same as that observed from noise exposure during the sensitive period. The sensitive period to ototoxic trauma is difficult to precisely determine, but the initial evidence suggests that it precedes the sensitive period to acoustic trauma in the mouse and rat. The observation of a difference in sensitive periods is important and needs further elaboration, since it suggests different underlying developmental mechanisms for these two traumatic agents. As a final point there may be a sensitive period for ototoxic exposure in the fetal guinea pig. Drug delivery to the mother is very effective in altering the fetal auditory system during the last three weeks of pregnancy (*23*).

Conclusions

The data in rat, mouse and hamster indicate that the sensitive period of greatest susceptibility to acoustic trauma occurs around that time that the cochlea is achieving its final stages of maturation. If this observation can be generalized to all mammalian ears, then the cochlea of man may be susceptible to acoustic trauma as it reaches its final stages of development between the eighth and fifth week before birth. It must be strongly emphasized, however, that there is not a single piece of direct evidence, that the authors are aware of, in support of this conclusion for man. Furthermore, the situation in placental mammals is complicated because the attenuating properties of the acoustic pathway to the fetal ear are poorly understood. The attenuation of the abdominal wall and placental fluids, and the fact that the middle ear is fluid filled, preclude the possibility of accurately specifying the effective stimulus reaching the fetal ear. These arguments, of course, do not apply to the premature infant born 5-8 weeks early. Similarly, antibiotic drug treatment of the mother during the last trimester of pregnancy may have a profound influence on the cochlea of the developing fetus.

This work was supported in part by a research award from the Deafness Research Foundation.

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Environmental Health Perspectives
Vol. 44, pp. 67-75, 1982

Clinical Assessment of Auditory Dysfunction

by W. G. Thomas*

Many drugs, chemicals substances and agents are potentially toxic to the human auditory system. The extent of toxicity depends on numerous factors. With few exceptions, toxicity in the auditory system affects various organs or cells within the cochlea or vestibular system, with brain stem and other central nervous system involvement reported with some chemicals and agents. This ototoxicity usually presents as a decrease in auditory sensitivity, tinnitus and/or vertigo or loss of balance. Classical and newer audiological techniques used in clinical assessment are beneficial in specifying the site of lesion in the cochlea, although auditory test results, themselves, give little information regarding possible pathology or etiology within the cochlea. Typically, ototoxicity results in high frequency hearing loss, progressive as a function of frequency, usually accompanied by tinnitus and occasionally by vertigo or loss of balance. Auditory testing protocols are necessary to document this loss in auditory function.

Introduction

Clinical assessment of auditory dysfunction is a broad topic, potentially covering many different methods of assessment and many types of auditory dysfunction. It is well known that auditory dysfunction can be caused by literally hundreds of different problems or pathologies. Auditory dysfunction can occur as a result of peripheral or central pathologies, or it can occur as a local manifestation of some systemic disease. Auditory dysfunction can be caused by extrinsic factors (infections, drugs, trauma, tumors, neurologic diseases, metabolic diseases) or intrinsic factors (genetic, etc.). Auditory dysfunction may be congenital or acquired. If congenital, it may be genetic, with hearing loss occurring alone as in the case of Mondini dysplasia, occurring in the form of a syndrome with other abnormalities such as Usher's syndrome, or may occur as a chromosomal abnormality. It may also be congenital but nongenetic, with hearing loss occurring alone in the case of many ototoxic agents, or, occurring with a host of other abnormalities as in the case of maternal rubella, anoxia, bacterial infections and metabolic disorders. As indicated, auditory dysfunction may also be acquired, either genetic or nongenetic. If genetic, hearing loss may occur alone as in the case of familial progressive sensorineural deafness or in conjunction with other abnormalities as in the case of Alport's syndrome. If nongenetic, hearing loss may occur as a result of numerous pathologies, including inflammatory diseases, ototoxicity, neoplastic disorders, traumatic injury, metabolic disorders, vascular insufficiency, or central nervous system diseases such as multiple sclerosis.

It should be obvious from this short description that the exact cause of a specific auditory dysfunction is frequently impossible to determine. It is also of interest to note that toxic agents may cause congenital or acquired auditory dysfunction. Since this conference is concerned with target organ toxicity—in the present case, specifically the auditory system—the remainder of this paper will deal especially with this topic. It should be remembered, however, that the same clinical assessment is appropriate to a wide range of auditory dysfunctions and not specific to those caused by ototoxicity.

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Ototoxicity

It is probably safe to say that the action of any substance on an organ or cell can be either beneficial or detrimental, depending on many factors. Some controversy continues, however, as to whether any chemical, drug or agent has a beneficial effect on the inner ear. Recent literature would indicate that nearly 200 substances have some documentation in the medical literature of ototoxicity (*1-3*). Although these cannot be described within the current space constraints, they can be separated into general categories. These include: chemicals (carbon monoxide, alcohol, nicotine, arsenic, potassium bromate, etc.); heavy metals (lead, tin, gold, mercury, etc.); antibiotics; diuretics; analgesics and antipyretics (salicylates, quinine, etc.); antineoplastics (bleomycin, nitrogen mustard, *cis*-platinum); and miscellaneous drugs (pentobarbital, hexadine). The literature on chemical or drug ototoxicity for antibiotics and diuretics is extensive. The aminoglycosides have received the majority of attention; however, other antibiotics, such as vancomycin, erythromycin, chloramphenicol, polymyxin B and ampicillin, have also been indicated as potentially ototoxic. Diuretics have also received substantial research as potentially ototoxic. Specifically, furosemide and ethacrynic acid have ototoxic properties, or at least potentiate ototoxicity. However, other diuretics such as mannitol and the mercurials may be ototoxics. Perhaps the greatest body of literature with regard to ototoxic effects concerns exposure to noise. Auditory dysfunction following noise exposure has been documented for more than 100 years.

It is an understatement and oversimplification to describe the action of any chemical, drug or other agent as complex, with potential ototoxic effects dependent on many variables. However, this is also an accurate statement. The mechanisms of action of ototoxic substances or agents may injure or embarrass the entire organ, specific cells within the organ, components of specific cells, or inhibit individual biochemical pathways. This action has been related to overall dose; duration of exposure; blood serum levels in the case of chemicals, drugs or heavy metals; general health of the subject and underlying disease; age of subject; prior exposure; renal impairment; individual susceptibility; and, of course, possible potentiation and synergistic effects of combinations of chemicals, drugs and other agents. All of these factors create a rather confusing picture with regard to true ototoxic effects and may explain, to some extent, apparent disagreements within the literature.

Auditory dysfunctions resulting from ototoxic agents usually present as tinnitus, hearing loss and/or vertigo. Cochlear damage is usually an orderly progression of hair cell loss in the organ of Corti beginning in the basal turn and progressing toward the apex. There appears first to be a consistent destruction of the inner row of outer hair cells, progressing to the outer two rows with preservation of the inner hair cells until overwhelming toxicity occurs and eventual total destruction of the organ of Corti. Injury to other cochlear structures has been demonstrated, including: changes in the stria vasularis, suprastrial portion of the spiral ligament, pericapillary tissues of the spiral prominence, outer sulcus cells, and Reissner's membrane. In addition, vestibular damage may occur, specifically in the Type I hair cells of the cristae ampullaris and in the utricle (*4, 5*). Auditory dysfunction resulting from ototoxic agents is almost always sensorineural. In most cases, this loss is bilateral and symmetric, although there are exceptions reported in the literature. Hearing loss caused by chemical or heavy metal toxicity may show brain stem or central pathology and is usually associated with a variety of other neurologic manifestations.

Clinical Test Battery

Clinical assessment of auditory dysfunction follows an orderly progression of tests to identify the site of disorder. While the shape of the auditory function may give information regarding possible pathology or etiology, auditory tests are designed to give primary information as to site-of-lesion. For example, auditory tests may indicate that the primary source of pathology is in the cochlea. These results, however, give no indication as to type of pathology (i.e., hair cell damage, damage to stria vascularis, rupture of Reissner's membrane, etc.), nor do they give information regarding possible etiology, since many factors, including ototoxicity, can cause damage to the cochlea. As a point of major differentiation, auditory tests are designed to separate peripheral and central auditory disorders. The major point of demarcation between peripheral and central disorders is the synapse at the cochlear nucleus, between the first- and second-order neurons. With this definition, pathologies in the external ear, middle ear, inner ear, and auditory nerve would be considered peripheral disorders and pathologies in the brain stem and cortex would be considered central disorders. Other auditory tests are designed to help differentiate site-of-lesion within the peripheral or central auditory system. As defined, auditory dysfunction in the peripheral system might be expected to show the following characteristics: ipsilateral symptoms, usual loss of sensitivity, distortion present in cochlear and auditory nerve disorders, and abnormal adaptation or fatigue in cochlear and auditory nerve disorders. Central auditory dysfunction might be characterized by contralateral symptoms, frequently normal sensitivity, and dysfunction in ability to transmit complex stimuli. Differences in characteristics of peripheral and central auditory dysfunctions have led to development of different auditory test batteries. In peripheral testing, pure tone stimuli can conveniently be used. Central testing, however, usually requires more subtle changes in stimuli and the use of complex stimuli (*6*).

The classical peripheral auditory test battery contains tests to differentiate among conductive, cochlear and retrocochlear hearing loss. In addition, tests are available to aid in differentiating sites-of-lesion within these three major areas. A complete description of auditory tests may be found in several standard audiology textbooks (*6-8*). A typical test battery might include the following tests.

Pure Tone Thresholds (Air Conduction and Bone Conduction)

Pure tone air conduction testing is used to measure the sensitivity of the ear to different frequencies, when compared to normal hearing. Air conduction testing uses the entire auditory system [i.e., external ear, middle ear, inner ear, eighth cranial nerve (N VIIIth), and central nervous systems]. Pure tone bone conduction testing is used to measure the sensitivity of the ear to different frequencies when the external ear and middle ear have been by-passed and sound is transmitted directly to the inner ear, although this is an oversimplification. The difference between air and bone conduction scores is the first evidence of conductive or sensorineural hearing loss.

Speech Reception Threshold (SRT)

SRT involves measurement of the sensitivity of ear to speech material. The speech materials generally used are spondee words (i.e., two syllable words with equal emphasis on each syllable, like baseball or airplane). The speech reception threshold should agree with the average pure tone air conduction results at 500, 1000 and 2000 Hz.

Speech Discrimination (SD)

Speech discrimination measures the ability of a subject to transmit and understand complex stimuli (speech). Speech discrimination lists are presented at a level above threshold so that they can be heard without difficulty, usually 40 dB SL, and the patient's ability to discriminate is measured. The speech materials generally used are short, single syllable, phonetically balanced words.

Alternate Binaural Loudness Balance (ABLB)

ABLB measures the presence or absence of loudness recruitment by having the subject balance the loudness of a standard tone in one ear with an alternating tone in the pathologic ear. The presence of loudness recruitment is measured by the growth of loudness in the pathologic ear. One contraindication of this test is that it cannot be used in bilaterally symmetrical hearing loss.

Short Increment Sensitivity Index (SISI)

The SISI test measures the ability of a subject to detect a 1 dB change in intensity at a level 20 dB above threshold. This test can be used at any frequency in either ear, regardless of the asymmetry of the hearing loss. A positive SISI score (i.e., above 60%) is an indication of cochlear pathology. Several modifications have been made to the classic SISI procedure (*9*).

Tone Decay (TD)

Tone decay measures the fatigue or adaptation of the auditory system to a constant stimuli. Tone decay is measured as the decay in decibels from threshold over a one minute period. A positive tone decay (i.e., greater than 25 dB) is an indication of N VIIIth disorder. Modifications to this original technique have been reported in the literature (*10-12*).

Bekesy Tracings

The Bekesy tracings indicate threshold sensitivity measured on an automatic audiometer when the tones are pulsed and when they are continuous. Theoretically, the pathologic auditory system should show more adaptation to a continuous tone than to a pulsed tone. Particular patterns of tracings have been identified with cochlear pathology and with retrocochlear pathology. The automatic Bekesy audiometer is essentially under the control of the subject. The subject is instructed to press a button when he hears a tone and release the button when the tone disappears. In this way, the patient automatically traces his threshold for pulsed and continuous tones. Thus, the audiometer can be used in several different ways.

Sweep Frequency. The audiometer automatically sweeps through frequencies from 100 Hz to 10,000 Hz, with the subject controlling the intensity. Pulse tones are used first, then continuous tones are plotted on the same audiogram, showing the amount of adaptation between pulsed and continuous tones (*13*).

Fixed Frequency. The audiometer can be set for one particular frequency and threshold for pulsed and continuous tones are plotted as a function of time. The purpose is to look at the amount of adaptation occurring at one particular frequency (*13*).

Backward Sweep. The audiometer can also be swept from 10,000 Hz to 100 Hz, showing whether the adaptation is a function of time or frequency (*14*).

Most Comfortable Loudness. The subject is instructed to keep the tone at his most comfortable loudness, which makes this a suprathreshold test. This modification is based on the concept that abnormal adaptation first appears only at high intensities and eventually appears at lower levels, until it finally appears at threshold. Abnormal decay of a continuous tone at suprathreshold levels is an indication of N VIIIth pathology (*12*).

Impedance

The study of impedance in the auditory system involves an analysis of the acceptance or rejection by this system of the flow of energy per unit of time. In other words, how much is the flow of energy impeded by this particular system? A system with high impedance rejects or reflects the majority of energy, while a system with low impedance accepts or absorbs most of the energy and reflects less. Normally, there are three components which combine to determine the impedance of a particular system: resistance, stiffness, and inertia or mass. In order to accomplish this clinically, a probe is placed in the external canal and sealed. The probe emits a low frequency tone (220 Hz), and a microphone in the probe measures the reflected sound from the tympanic membrane. The amount of sound reflected gives an indication of the integrity of the external and middle ears. Normally, four measures may be made—static compliance, tympanometry, acoustic reflex threshold and acoustic reflex decay.

Static Compliance. Static compliance of the middle ear is a measure of mobility. Mass (inertia), resistance (friction) and stiffness (or its reciprocal compliance) work together in a complex manner to facilitate, or impede, motion of the middle ear system, as measured at the tympanic membrane (MT). Historically, static compliance has been termed acoustic impedance or absolute impedance, although the term compliance is a more descriptive term of what is actually measured. Static compliance denotes a single number representing the mobility of the middle ear system. Tympanometry is also a measure of compliance; however, this measure is made over numerous values as the MT moves in response to changes in air pressure and, thus, is a measure of dynamic compliance.

Static compliance of the middle ear system is measured by quantifying the sound energy reflected from the MT. When the middle ear system is stiff, more energy is reflected rather than absorbed or transmitted through the middle ear. Therefore, a stiff middle ear system is said to have low compliance or high resistance. A flaccid middle ear mechanism absorbs more energy and reflects less. Therefore, this system has high compliance or low resistance.

Static compliance can be measured in terms of equivalent volume of air in cubic centimeters or in acoustic ohms. This test requires two measurements: one measurement made with the MT in a position of low compliance by exerting an air pressure of +200 mm of water in the external ear relative to the middle ear; the second measurement is made with the MT in a position of maximum compliance (normally at 0 mm of water). Neither of these two measures has any significance when taken alone. However, by subtracting one measure from the other, the external ear canal volume is effectively cancelled, thus allowing a measurement value of the middle ear mechanism. The compliance of the normal middle ear system is influenced by many variables, including age and sex. In general, however, normal static compliance values range between 0.26 and 1.5 cc. A stiff middle ear system should demonstrate a compliance value less than 0.26 cc, while a flaccid middle ear system should have a compliance greater than 1.5 cc. The normal range of absolute impedance in acoustic ohms is approximately 600 to 3000 ohms. Absolute values below 600 ohms indicate a very compliant ear, while values above 3000 ohms indicate a resistive middle ear system.

Tympanometry. Tympanometry is the measurement of eardrum compliance as the air pressure is altered in the external ear relative to the middle ear. These measurements are normally recorded on a graph which represents a compliance–air pressure function, called a tympanogram. A point of significance is that the MT is at maximum compliance when the air pressure in the middle ear is equal to that in the external ear. Tympanometry can provide an indirect measure of existing middle ear pressure by identifying the air pressure in the external canal, where the eardrum shows its maximum compliance. Subjects who have intact MTs, with no middle ear pathology and adequate eustachian tube function, will show maximum compliance at atmospheric pressure or within ± 50 mm of atmospheric pressure. Subjects with intact MTs and poor eustachian tube function will show maximum compliance at negative air pressure values. Subjects with fluid in the middle ear will usually never reach a point of maximum compliance, to the maximum of the instrumentation, while those with a resistive type middle ear pathology will show very low compliance (high stiffness or resistance) at atmospheric pressure.

Acoustic Reflex Threshold. The stapedial muscle contracts reflexively when the ear is stimulated with a sufficiently loud sound. In normal ears, the acoustic reflex can be elicited with stimulation at sensation levels of 70 to 95 dB. Contraction of the middle ear muscles decreases the compliance of the MT. This contraction occurs bilaterally. In the demonstration of the acoustic reflex, the sudden change in the relative compliance of the middle ear created by the muscle contractions is utilized. If the acoustic signal is sufficiently loud to elicit the bilateral acoustic reflex, the resulting contraction of the stapedius muscle in the probe ear will suddenly decrease the compliance at the MT synchronously with the presentation of the stimulus and this change in relative compliance can be observed as a sudden deflection in the balance meter. The acoustic reflex can be elicited by either contralateral stimulus presentation or ipsilateral stimulus presentation at approximately the same stimulus levels. Also, the reflex may be elicited with pure tone stimuli or with noise.

Reflex Decay. Reflex decay is a truly remarkable phenomenon. In normal ears, contraction of the middle ear muscles to an auditory stimulus of 1000 Hz or lower can be maintained for up to 45 sec without obvious decay, fatigue or adaptation. In subjects with retrocochlear lesions and some cochlear lesions, the reflex appears normal when first turned on. When the acoustic stimulus is sustained, however, reflex amplitude declines and may eventually disappear. Klockoff and Anderson et al. (*15, 16*) advocate the use of reflex decay as an indicator of VIIIth nerve lesions. These authors have reported a decay of the acoustic reflex for patients with VIIIth nerve pathology when a stimulus is presented at a reflex sensation level of 10 dB for 10 sec. When a stimulus is presented in this manner, the amplitude of the reflex decays to a level of 50% or less of the original amplitude in less than 10 seconds. The same results have been found in normal ears at 4000 Hz, and to a lesser degree at 2000 Hz. No decay is observed in normal ears at 500 and 1000 Hz, however.

PI-PB Function

The PI-PB (performance versus intensity for phonetically balanced words) function test, a special use of speech discrimination, simply refers to the discrimination test given at several different intensities. In the case of cochlear pathology, the function should reach a plateau with increased intensity and remain there, while in VIIIth nerve pathology, the discrimination score becomes worse at high intensities (*17*).

Central Auditory Dysfunction

Tests for central auditory dysfunction will not be discussed in this presentation. The reader is referred to several excellent texts describing these tests (*18, 19*). The majority of tests used to identify central auditory dysfunction use complex stimuli like speech because changes in the central nervous system are usually subtle. In general, the speech material is changed in some way to reduce its redundancy and increase its ability to detect subtle changes. Tests have been designed which alter speech stimuli in many ways. These include: submerging the speech material in a background of noise; combining the speech material with a competing message of different speech material, either in the contralateral or ipsilateral ear; filtering the speech material to reduce its intelligibility; interrupting the speech material; accelerating the speech material; presenting different frequency bands of speech to different ears; and, presenting two different speech messages simultaneously to the two ears (dichotic speech). Some toxic substances effect the central nervous system more than the peripheral system. These include carbon monoxide and heavy metals. In these cases, complete clinical assessment would include central auditory tests.

Brain Stem Tests

Several more recent auditory tests appear to show promise in enhancing the clinical assessment of auditory dysfunction. The most prominent appears to be the auditory brain stem evoked response (BSER, BER or ABR). This is one of several evoked responses that have been identified in the auditory system. At present, the following responses have been identified: auditory nerve response (latency 1-4 msec); brain stem or fast responses (latency 2-12 msec); middle responses (latency 12-50 msec);

sonomotor or muscle responses (latency 10-50 msec); slow auditory responses (latency 50-300 msec); and contingent negative variation (CNV) (latency 300-600 msec).

One of the more stable evoked potential measures is the brain stem response. This is a series of vertex-positive waves following a click or tone burst with latencies from 1 to 10 msec (*20-26*). To date, seven different waves have been identified and associated with various nuclei in the auditory brain stem system (*21*, *27*). The most prominent and visible wave of this series occurs with a latency of approximately 6 msec and is called the V wave (*28-30*). It is assumed to originate in the inferior colliculus and is a good candidate for assessing the higher auditory frequency responses occurring in the basal turn of the cochlea. Other waves have been ascribed to the auditory nerve (wave I), cochlear nucleus (wave II), trapezoid body (wave III), lateral lemniscus (wave IV) and medial geniculate (wave VI). The utility of the brain stem response as a clinical tool seems to be enhanced since the response is extremely stable, is not unusually affected by state of sleep, can be recorded from unconscious patients, shows a maturational development, can be recorded in a relatively short period of time and includes waves which are associated with various nuclei in the auditory system of the brain stem (*31*, *32*).

The latencies of the various waves of the brain stem evoked response are extremely stable from test to test in the same subject and between subjects. In fact, the standard deviations around these mean latencies range from 0.1 to 0.3 msec (*33*, *34*). In addition, the latency changes in a predictable manner as the intensity of the stimulus is increased or decreased. This latency versus intensity relationship makes the BSER a valuable tool in assessing hearing function of difficult to test subjects. For example, the mean latency of the V wave in normal hearing subjects at 70 dB above threshold is approximately 5.5 msec. As intensity is decreased to 10 dB above threshold, the latency increases to approximately 8.0 msec (*35*). Therefore, a measure of the latency of the V wave in a difficult to test subject would give some estimate of the subject's threshold for that particular stimulus. In addition, the latency of the various waves of the BSER, particularly the V wave, have been shown to exhibit recruitment (*35*). The presence of this phenomenon can give a good indication as to whether the pathology is of cochlear origin or not. It would appear that this technique holds considerable promise in evaluating performance with hearing aids in difficult to test subjects or subjects too young to respond in conventional manner. It would seem possible to predict the gain of the amplification system by measuring the latency of the V wave with and without amplification.

Another important aspect of the BSER is the neural transmission time. This is a measure of the latency between the I wave (i.e., primary auditory nerve) and the other waves, giving a measure of the travel time in the auditory brain stem system. This measure has proved valuable in assessing the site of pathologies in the brain stem, such as acoustic tumors, multiple sclerosis and brain stem vascular and neoplastic lesions. In very young children, this transmission time is also delayed, probably due to incomplete maturation or myelination of the central auditory pathways. This latency reaches normal adult values, however, around one year of age. Increased neural transmission time in older children or adults with normal latencies for the I wave may be an indication of lack of maturation, demyelinating pathologies or the presence of space occupying lesions, etc.

Another potentially important addition to the auditory test battery for clinical assessment is the use of high frequency audiometry. This measurement of auditory function in the frequencies from 8000 Hz to 20,000 Hz has potential significance to both clinical and research testing. Changes in high frequency auditory thresholds have been described as an early indication of ototoxic effects of certain drugs and noise exposure (*36-38*). This procedure has been used by several investigators; however, it is not yet completely accepted as a clinical measure because of difficulties in instrumentation (*39-41*). This procedure, with adequate and stable instrumentation, could serve as a valuable function in detecting early auditory dysfunction.

Clinical Assessment

As previously stated, clinical assessment of auditory dysfunction usually includes a progressive battery of tests designed to indicate site-of-lesion. The first order of priority is to determine the amount of auditory dysfunction, if any, and the extent of this dysfunction. This is normally accomplished with pure tone thresholds for various frequencies, speech reception thresholds, and speech discrimination scores. Assuming an auditory dysfunction is present, the second order of priority is to determine if this dysfunction is conductive, sensorineural, mixed or central. A mixed-type hearing loss has components of both conductive and sensorineural origin, since these types of hearing losses are not mutually exclusive. This is normally accomplished with pure tone bone conduction thresholds and the impedance test battery. For a conductive hearing impairment, pure t[illegible] bone conduction thresholds should be normal [illegible] ear normal in the presence of abnormal pure to[illegible] ir conduction thresholds. Speech reception th[illegible] olds should agree within ± 10 dB of averag[illegible] onduction thresholds at 500, 1000 and 2000 H[illegible] l speech discrimination should be normal (9[illegible]. The impedance test battery should show [illegible] al static compliance (either resistive or com[illegible] depending on the nature of the hearing los[illegible] panometry should be abnormal (showing a v[illegible] mpliant system, a very resistive system, n[illegible] e pressure or the presence of middle ear eff[illegible] s, depending on the site and type of dysfunc[illegible]. acoustic reflex should be absent (depending [illegible] xtent of conductive hearing loss), and acoustic [illegible] ex decay will obviously not be measured if [illegible] reflex is absent. Reports in the literature ha[illegible] dicated that a very mild conductive hearing [illegible] will cause the absence of the acoustic reflex in [illegible] e affected ear (*42*). At this point, a specific treatment protocol is indicated since the majority of conductive hearing losses can be medically or surgically treated. Also, there is no concrete evidence of conductive hearing losses resulting from ototoxicity.

Should auditory tests indicate a sensorineural hearing loss, the third order of priority is to delineate between cochlear and retrocochlear involvement. In the case of sensorineural hearing loss, pure tone air conduction thresholds should be abnormal, although they may vary from normal to total deafness in retrocochlear lesions. Pure tone bone conduction thresholds should agree reasonably well with air conduction, indicating no involvement of the external or middle ear. Speech reception threshold should agree with average pure tone thresholds and speech discrimination will usually be abnormal. In the [illegible] ochlear involvement, speech discrim[illegible] ill vary between 50 and 90%, while [illegible] of retrocochlear involvement to [illegible] rimination scores much lower than 90[illegible] dance test battery will usually show specific [illegible] of patterns for cochlear and retrocochlear [illegible] vements. In both cases, static compliance an[illegible] mpanometry should be normal since there [illegible] mably no middle ear involvement. Cochlear [illegible] vement, usually accompanied by loudness re[illegible] ent, will normally show the presence of an a[illegible] e reflex if the hearing loss does not exceed [illegible] 85 dB. One indication of cochlear involve[illegible] is the presence of acoustic reflexes at abnorm[illegible] low levels (*42*). If the reflex is present in cochl[illegible] hearing loss, it will normally show no significa[illegible] decay. Retrocochlear lesions, on the other hand, w[illegible] frequently present with an absence of reflex, e[illegible] n in the presence of sufficient hearing to elicit this reflex. If the reflex is present in retrocochlear lesions, it usually decays abnormally.

The use of the classic diagnostic test battery is also helpful in distinguishing between cochlear and retrocochlear lesions. Usually the tests which indicate an abnormal sensitivity to changes in loudness (ABLB, SISI, and to some extent the Bekesy) will show abnormal results in cochlear lesions. The ABLB will normally show the presence of loudness recruitment in the affected ear and the SISI will show a high percentage of small intensity increments detected (greater than 60%). In classical retrocochlear lesions, these tests are usually normal, since abnormal sensitivity to loudness changes is associated with cochlear pathology. Tests which show abnormal adaptation, such as the tone decay and to some extent the Bekesy, will show no abnormal tone decay (less than 25 dB) and a Type II Bekesy tracing. Retrocochlear lesions will normally indicate abnormal fatigue or adaptation on the tone decay (greater than 25 dB) and show Type III or Type IV Bekesy tracings. The performance versus intensity function for speech discrimination (PI-PB) also shows unique results for cochlear and retrocochlear lesions. For cochlear lesions, this function will normally reach a maximum discrimination and plateau or show a very slight decline. For retrocochlear lesions, however, this function will usually reach a maximum, although at a low discrimination level, and show a severe decline or roll-over as intensity is increased.

The brain stem evoked response has become one of the most reliable tests in differentiating cochlear and retrocochlear lesions. In conductive hearing losses, the results are often quite variable, since it may be difficult to present stimuli at sufficient levels to elicit adequate responses. In cochlear lesions, especially those exhibiting loudness recruitment, BSER results are usually normal and may even show a decreased latency when compared to the level of stimulation. In retrocochlear lesions, the BSER usually indicates abnormal latency measures, especially for waves III, IV and V. This test is obviously useful in indicating other types of lesions in the brain stem which might alter amplitude of response or latency of response. These may include space occupying lesions and demyelinating lesions. Table 1 shows the relationship between various auditory tests used in clinical assessment and their expected results in conductive, cochlear, and retrocochlear pathologies.

As indicated earlier, the majority of ototoxic substances or agents have their direct effect on various parts of the cochlea, with the noted exceptions. While clinical assessment cannot differentiate between various pathologies or etiologies affecting

Table 1. Hearing tests used in clinical assessment and expected results for conductive, cochlear and retrocochlear lesions.

	Type of hearing loss		
	Conductive	Cochlear	Retrocochlear
Pure tone thresholds			
Air conduction	Abnormal	Abnormal	Variable
Bone conduction	Normal	Abnormal	Variable
Speech reception threshold	Abnormal	Abnormal	Variable
Speech discrimination	Normal	Abnormal	Abnormal
Impedance battery			
Static compliance	Abnormal	Normal	Normal
Tympanometry	Abnormal	Normal	Normal
Reflex threshold	Absent	(?) Abnormal	Absent/Decay
Reflex decay	N/A	Normal/Absent	Absent/Abnormal
Diagnostic battery			
ABLB	Normal	Abnormal	Normal
SISI	Normal	Abnormal	Normal
Tone decay	Normal	Normal	Abnormal
Bekesy	Type I	Type II	Type III or IV
PI-PB	No rollover	Plateau	Rollover
Brain stem evoked response	Variable	Normal/Variable	Abnormal

the cochlea, results should indicate cochlear pathology in the presence of ototoxic substances or agents. The shape of the audiometric function frequently can give an indication of toxic damage to the inner ear. Noise exposure, for example, usually has a characteristic audiogram with the greatest loss at 3000, 4000 or 6000 Hz, while ototoxicity from drugs or chemicals shows a progressive hearing loss as a function of frequency, frequently severe in nature. This hearing loss is usually accompanied by tinnitus and occasionally by vertigo.

In clinical assessment of auditory dysfunction, including that caused by ototoxicity, several points should be made. In this section, descriptions such as frequently, usually and normally are used repeatedly. This has not resulted from lack of vocabulary, but because of the variability in auditory tests. Review of the pertinent literature indicates that any single auditory test, taken alone, shows the classic result in 60–90% of cases (*6, 43-45*). This means that a relatively high percentage of subjects (10-40%) may show retrocochlear signs on some tests with cochlear lesions, or, cochlear signs in the presence of retrocochlear lesions. In addition, the nature of the lesion itself may cause damage in more than one anatomical area. An acoustic tumor, for example, which should indicate a retrocochlear lesion, may also compromise the blood supply to the cochlea, causing cochlear damage. In this case, the test results would be quite variable and show indications on various tests of both cochlear and retrocochlear lesions. The first point, therefore, is that the entire test battery should be considered in the assessment with less weight to the results of a single test. The percentage of false positive and false negative results drops significantly when expected results are found on two, three or four of the tests in the battery. The second point is that test results should be interpreted in conjunction with a detailed case history. While an auditory test battery may give an indication as to the site of the lesion, the case history and other pertinent data may shed light on the possible etiology.

The third major point in clinical assessment of auditory dysfunction, particularly in the case of ototoxicity, is the development of adequate testing protocols. Toxic effects on the human auditory system are quite variable and the presence and extent of damage depends on many parameters. Toxic effects may occur immediately, as in the case of many diuretics, occur within days, with various drugs and chemicals, or occur over years, in the case of heavy metal toxicity and noise exposure. In addition, decrease in auditory function may continue for months after the toxic substance has been removed. Therefore, hearing assessment to detect changes in auditory sensitivity should be on a regular basis, depending on the substance or agent involved. In the case of aminoglycosides, diuretics, certain other antibiotics, and antineoplastics, testing should probably occur on a weekly basis while the subject is on the drug and monthly, up to six months, after cessation of the drug. In the case of analgesics and antipyretics, monthly to quarterly testing is probably adequate. Exposure to noise or heavy metal toxicity may require annual testing, although testing for heavy metal toxicity may depend on the blood levels of the heavy metal. It is also obvious that pre-exposure assessment would be ideal, to separate changes in auditory function attributed to the ototoxic agent from pre-existing auditory dysfunction and to serve as a baseline for comparison of changes in auditory function. However, in many cases of life-threatening illness or infection documented decreases in auditory function resulting from the therapy may be purely academic.

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Environmental Health Perspectives
Vol. 44, pp. 77-85, 1982

Concerning The Need for More Sophisticated Animal Models in Sensory Behavioral Toxicology

by William C. Stebbins*

It is necessary but not sufficient to develop laboratory animal models in sensory behavioral toxicology for screening toxic substances and for the analysis of sensory impairment at threshold levels of stimulation. It is important to develop more thorough and quantitative tests of impairment which in their greater complexity more accurately reflect the conditions and environmental demands of day-to-day life. Such greater complexity in stimulus conditions and behavior may also aid in monitoring not merely the state of the receptor organ but more central nervous processes which are the focus of assault by many known toxic substances. Techniques are described for studying such acoustic behaviors as intensity discrimination and frequency selectivity in guinea pig and monkey by use of operant conditioning procedures coupled with sensory testing (psychophysical) methods. Impaired auditory selectivity and discrimination is shown to be correlated with histopathological changes in the inner ear. Slight modification of these procedures in animals may be used to investigate acoustically more intricate behaviors such as sound localization and the perception of frequency modulated acoustic signals as elements of speech and communication sounds.

One class of animal models in the study of behavioral toxicology has an obvious appeal because of its simplicity. The canary in the coal mine is an old and familiar example: it was an all-or-nothing proposition. If the carbon monoxide in the mine reached a certain level, the canary died, and it was time for the men to get out of the mine. This was not, however, a model which invited further and more detailed analyses of the behavioral effects of the toxic substance. The canary did not stop singing, for example, at lower levels of carbon monoxide, nor were there observed changes in other forms of canary behavior as a consequence of the pollutant. It provided a simple, reasonably effective model for behavioral toxicology but one with a somewhat limited scope.

To better understand the effects of toxic substances on behavior and the toxic process, we need a more informative canary—one that can tell us not only that things are getting bad before they have gone beyond the point of no return, but also one that tells us where it hurts so that we can discover specific processes and physiological mechanisms which may represent target sites for the toxic substance in question. Put in another way, we need a more sophisticated canary trained to answer in a quantitative fashion a number of very specific questions about the continually deteriorating state of its health brought on by exposure to some poison.

Clearly, animal models can serve a variety of very different functions in behavioral toxicology. For example, one essential approach involves the development of simple, highly analytic measures of behavior which are employed as baselines for the study of the effects of known or presumed poisons. Such behavioral measures as general activity, eating and drinking, seeing and hearing may be readily obtained, in some instances, from large numbers of animals, and may serve in the screening of a wide variety of toxic substances.

With regard to hearing, for example, we may be concerned simply with whether normal sound levels are audible to an animal without regard to the intensity or other characteristics of the acoustic signal. Observations of the pinna (ear flap) reflex, startle, or escape from a sound which foretells of impending electric shock all give us at least "rough and ready" measures of hearing in a more general sense. Few would question the need for such measures which may provide us with clear indications that something has gone awry in the environment. The advantages of such measures reside in the ease and relative quickness with which they may be obtained. Lengthy training time and complicated equipment are not necessary. Secondly, such measures can serve as an early warning system for the build-up of toxic substances in the environment and in the bodily tissues. Unfortunately, in return for speed and ease of training, some sacrifices must be made in the level of sophistication and complexity of the information obtained by these procedures and sometimes even in its validity and reliability.

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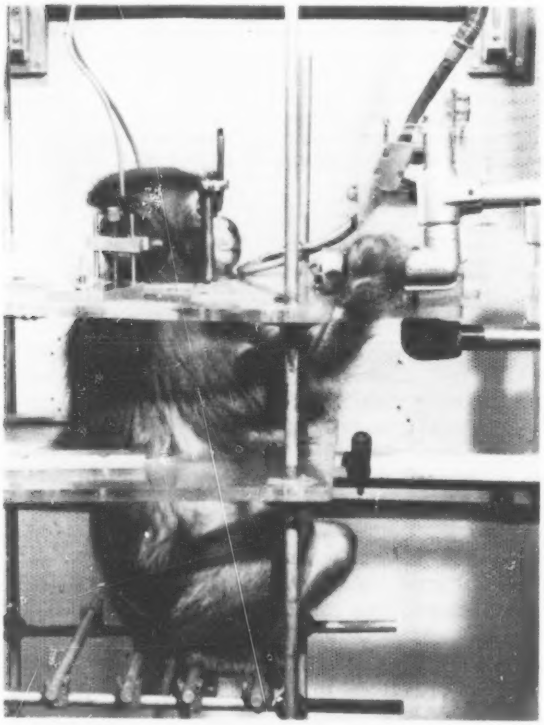

Figure 1. Subject, a macaque monkey, seated in restraining chair for hearing testing. Earphones, cylindrical response tube, and feeder tube are shown. The monkey is in listening position with his hand grasping the response tube prior to tone stimulation (*2*).

It becomes important to probe further once it is clear on the basis of the preliminary results obtained from these simpler screening procedures that some form of behavioral impairment has resulted from exposure to a toxic agent. Some form of scale is necessary which will relate the degree of impairment to the quantity of and duration of exposure to the poison. Its time course of action is equally important and makes this more than a simple dose-effect curve. Daily or even hourly monitoring will reveal the earliest signs of damage, but such extensive testing precludes mass screening with large numbers of animals. The hope is for a much more detailed behavioral profile of action of the toxin. To establish such a scale in an animal model requires lengthy behavioral training regimens and often fairly elaborate instrumentation. There is presently no viable alternative.

A particularly relevant example may be found in the threshold testing of monkeys by using simple operant conditioning methods with food reinforcement (*1*). In about two months, animals fresh from the jungle learn to report through a computer when an acoustic stimulus, briefly presented, is just audible. The monkey seated in a chair (Fig. 1) responds to a flashing light by touching a metal tube. Contact with the tube is maintained until the sound is presented through earphones. If the animal then removes its hand from the tube while the sound is on, reinforcement in the form of a small food tablet follows immediately. Time-out from the experiment—a delay in food availability—is a form of punishment given for responding when the sound is off. Thresholds are determined by reducing the intensity of the stimulus after it is correctly detected and increasing its intensity following the subject's failure to respond to it. A tracking method is used in which there is a feedback loop between subject

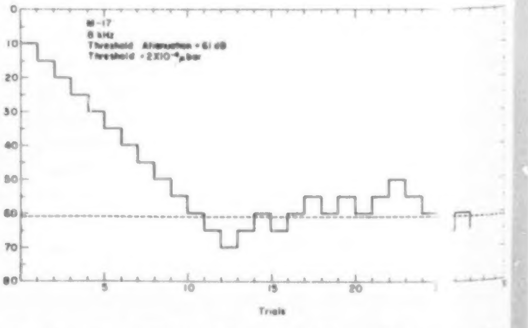

Figure 2. Use of the tracking method for audiometric testing of monkeys. Correct detections cause the tone to be attenuated in 5 dB steps, while failure to hear produces a subsequent 5 dB increase in tone intensity (*3*).

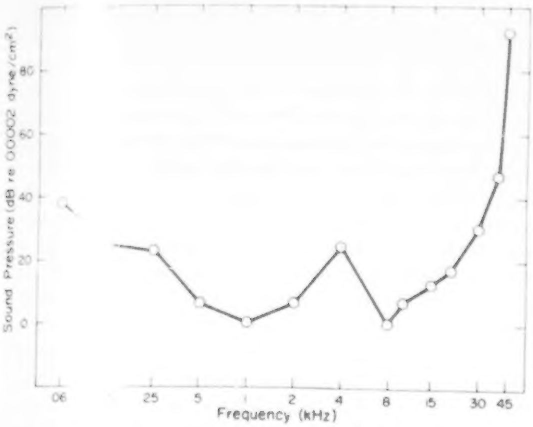

FIGURE 3. Threshold of hearing function for macaque monkey (2).

and stimulus; the record of responses to the stimulus resembles an up-and-down staircase (Fig. 2). Threshold represents the stimulus value to which the subject responds correctly 50% of the time. Such thresholds are determined over a wide range of frequencies resulting in a function like the one in Figure 3, in which sound intensity at threshold is shown to vary over the animal's audible frequency range.

Threshold or the minimum detectable level of acoustic stimulation is affected by a variety of poisons and environmental pollutants. Aging in man brings with it a loss in sensitivity to high frequencies. The aminoglycoside antibiotics produce a similar effect, although in a much briefer time period. The characteristic picture of progressive hearing loss is seen in an experimental animal treated with the antibiotic, kanamycin (Fig. 4). The inexorable progression of the impairment from high to low frequencies is evident in this example. At a certain point in time, treatment is discontinued, and the subject tested over a further time interval for any delayed effects of the drug on hearing. The animal is then sacrificed and its temporal bones taken for microdissection and histopathological analysis of the organ of Corti of the inner ear. The results together with the terminal threshold measurements are shown in Figure 5. There is a striking correlation between the extent and pattern of receptor cell loss along the basilar membrane in the inner ear and the high frequency hearing impairment. The receptor cells and perhaps their supporting cells and the fibers of the auditory nerve are clearly implicated as targets for the site of action of this ototoxic drug (4).

Thresholds for tonal stimuli at minimum detectable levels of stimulation provide an important measure of hearing and may even offer some insight into underlying mechanisms particularly, as just described, for those agents whose primary destructive focus is within the inner ear. Such threshold measures, however, are limited in terms of the information they offer about the overall performance of the auditory system.

A primitive function of the auditory system is its ability in detecting low level acoustic energy—heavy feet on the substrate. In insects, most fish and in our early land-dwelling vertebrate ancestors, detection was the primary if not the sole function of the auditory system. Its currency pur-

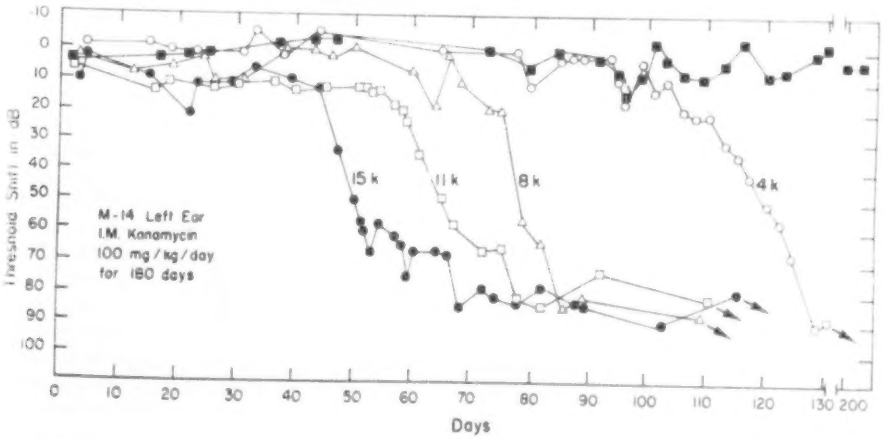

FIGURE 4. Progressive changes in threshold for one macaque monkey for different acoustic frequencies during daily kanamycin treatm The zero line represents normal hearing at all frequencies prior to drug treatment (4).

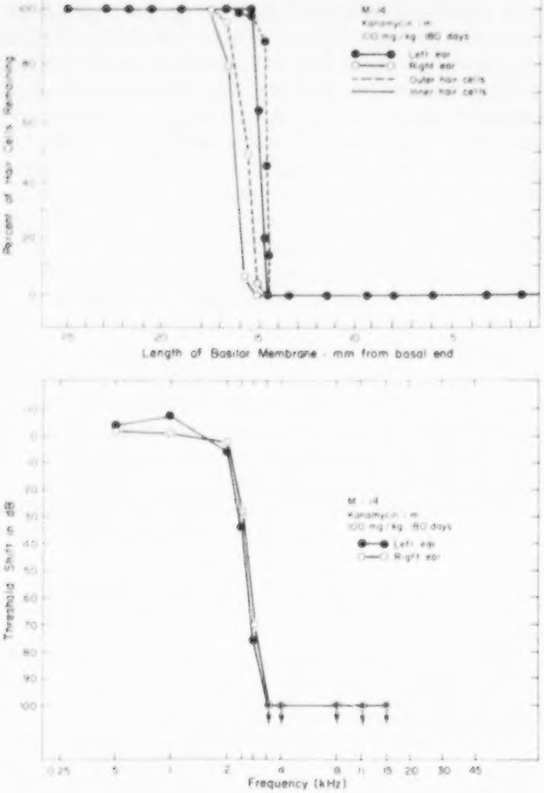

FIGURE 5. Auditory threshold shift for the monkey represented in Fig. 4 after 180 days of daily kanamycin (lower panel); hair cells remaining as a function of position on basilar membrane (upper panel) (3).

chased a mate, a meal, and on many occasions helped an animal avoid being included on someone else's menu. In the living mammals, particularly the primates including man, a more highly evolved auditory system plays a much more complex and diverse role. Beyond simply detecting acoustic events in, for example, an early warning mode, it is highly discriminating on the basis of acoustic frequency, intensity, time and timbre or acoustic complexity. Effective communication assumes that the normal listener is able to make fine discriminations in all these dimensions. The physical distinction between "bad" and "dad", for example, lies in the initial 50 msec frequency transition upward or downward depending on whether the first letter is a "b" or a "d". The putative higher vertebrates also are very accomplished in locating with considerable precision the source of a sound in three-dimensional space, a skill which has given them the edge over their less capable forebears in capturing prey, avoiding predation, and finding a mate.

In order to comprehend fully the normal or the impaired auditory system, it is essential to examine functions other than those involved in the simple detection of acoustic signals. The questions assume a higher level of complexity and, in so doing, approach more closely the state of affairs in the real world. Many signals, for example, are clearly audible to us, but it is often important that we be able to locate their source and accurately determine their direction of movement. These are biologically important activities in daily use and they assume an intricate interplay between the auditory system and the rest of the central nervous system. Deterioration in the skill with which these activities are performed implies something regarding the nature and location of the deficit in the nervous system. If, in addition, the responsible agents are neurotoxic substances, we stand to gain information regarding their locus and mechanism of action.

A broad spectrum of biologically relevant acoustic behavior should be considered for study and use as baselines for toxicological intervention. In addition to sound localization, these behaviors include differential acuity for acoustic frequency and intensity, discrimination of the complex acoustic signals used in speech and communication, judgment of loudness, and the ability to extract a signal from a noisy background. All are functions, which like sound localization, play a substantial role in our daily lives. Although their impairment is seldom life-threatening it may presage far more harmful consequences to other bodily systems as a result of continued exposure to the noxious substance.

Only recently have the conditioning techniques which have been applied successfully to the measurement of thresholds in experimental animals been extended to the analysis of more complex auditory functions. It is a major purpose of this paper to describe some of the experiments that have been carried out in order to demonstrate their potential for a full scale analysis of hearing and ototoxic hearing loss in animal models. Again, these are not procedures which lend themselves easily to the screening of large numbers of chemical substances in large numbers of animals. The procedures described here are arduous, time-consuming, and heavily instrumented. Their virtue lies in their ability to provide exact quantitative behavioral measures of a variety of hearing functions. Together with physiological, biochemical, and anatomical measures they can aid in pinpointing basic mechanisms and thus offer a logical and far more detailed follow-up of basic toxicological screens than simple threshold detection functions.

Operant conditioning procedures, together with sensory testing (psychophysical) methods, provide an effective means of questioning a nonhuman animal about the limits of its sensory experience. A slight modification in the basic threshold testing protocol described earlier permits the sampling and accurate measurement of a wide range of such experiences. For example, the ability to discriminate small differences in the various parameters of an acoustic signal (i.e., intensity, frequency, etc.) permits the animal or man to respond to the most subtle differences in communication sounds or language. Loss of this ability in the hearing impaired produces a distinct handicap and may or may not be correlated with a significant loss in detection threshold. In evaluating the limits of this ability, the subject is asked to report the smallest discriminable differences between two sounds (usually tones).

In a recent experiment (*5*), guinea pigs were trained to report such minimal differences in the intensity of a pure tone. Their pure tone detection thresholds were also determined. The animals were operantly conditioned to depress a small switch in the floor of their test cage in response to a flashing light. Depression of the switch initiated a trial which included a series of tone pulses at fixed intensity. After a brief but varying time interval a tone pulse of higher or lower intensity alternated twice with those pulses in the initial pulse train. If the animal responded to this change in sound intensity by releasing the switch, it was reinforced with food. Release of the switch at any other time was followed by punishment in the form of a time-out from the experiment. When the intensity difference was correctly reported by the animal, that difference was reduced on the subsequent trial; however, if the animal failed to report the intensity difference by continuing to depress the switch, the difference was subsequently increased. The testing procedure was a variation on the original threshold procedure described earlier and yielded a quantitative measure of intensity difference threshold as that difference correctly reported by the animal on 50% of the trials. Normal values obtained from several animals were found to be reliable and stable and only slightly larger than values obtained for human subjects.

The guinea pigs were then treated daily with the antibiotic kanamycin for about two months. Their hearing was assessed daily either by the conventional threshold procedure or by the intensity discrimination threshold method. Typical results are presented for one animal. (Fig. 6). In the lower function the detection threshold results are displayed. At this test tone frequency of 8 kHz, the animal experienced a rather sudden and precipitous hearing loss just prior to the end of the drug treatment. Although the hearing loss was considerable (about 70 dB), it was not complete. A particularly interesting finding, which was observed for all treated subjects, is seen in the upper function which represents the intensity difference threshold obtained during and after drug treatment for the same animal. The results merely reflect the stability of the measure over time, for no significant departure from baseline (at 0 dB on the figure) and thus no loss in intensity discrimination is evident throughout the treatment period or thereafter. Differential acuity for sound intensity was tested at sound levels well in excess of the threshold of hearing (70 dB) before drug treatment and thus only slightly above threshold (20 dB), which had been markedly altered, after drug treatment.

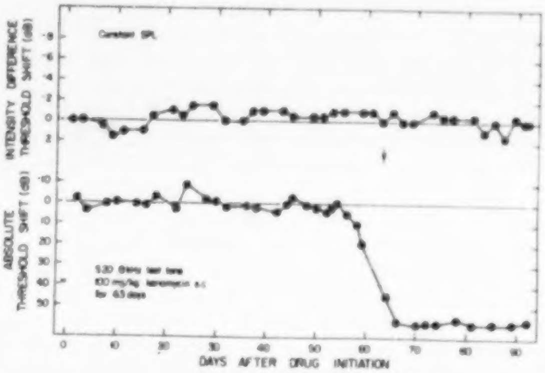

FIGURE 6. Intensity difference threshold shift (upper panel) and pure tone detection threshold shift (lower panel) at 8kHz as a function of days after initiation of treatment with kanamycin for one guinea pig. Arrow indicates cessation of drug treatment at 63 days (*5*).

These results have important implications for behavioral toxicology, for they clearly indicate that, as a consequence of exposure to a toxic substance, one function of a sensory system may be drastically altered while another, seemingly closely related function, remains intact. Of further interest are the subsequent histopathological findings in the inner ears of those treated guinea pigs. Of the two populations of morphologically different receptor cells in the organ of Corti, the outer hair cells were missing from the basal half of the cochlea in these animals while the inner hair cells remained intact and appeared normal and were probably functional. It would be premature to state at this point that the missing outer hair cells reflect the considerable threshold shift, while the inner hair cells still in place, play an important role in acoustic intensity discrimination. The findings are suggestive, but more importantly they exemplify the kind of conceptual issues which arise when behavioral toxicologists go beyond routine screening and simple detection measures. The basis for the effect of the toxic substance may now be explored more fully.

The ability to resolve small frequency differences in acoustic stimulation may be measured by a frequency discrimination paradigm similar to the one for intensity discrimination just described for the guinea pig. Another strategy involves the masking of one pure tone by another with the subject trained to respond when one tone, the test tone, is just audible above the second tone, the masker (*6*). A test tone, always of the same frequency and intensity (usually slightly above detection threshold), is presented together with the masker, a tone which is varied systematically in both intensity and frequency. Often the masker begins at a frequency well below that of the test tone; its intensity is adjusted by the tracking or staircase method and a threshold level of the masker is determined as that level that enables the subject to respond correctly to the test tone on 50% of the trials. The masker frequency is then changed and a new threshold for detection of the test tone is determined. The process is continued until an entire function—a psychophysical tuning curve—is generated. For animal subjects the conditioning procedure is a simple adaptation of the one described previously. The subject makes a contact response, holds, and then breaks contact when the test tone can be heard over the level of the masker. A psychophysical tuning curve for a monkey is shown in Figure 7. The test tone was at 2 kHz, 10 dB above the animal's threshold at that frequency. The masker frequency was varied from about 300 to 3000 Hz. A measure of tuning or frequency resolution is provided by the *Q* metric which is usually the test tone frequency divided by the frequency bandwidth of the function 10 dB above the tip. A family of psychophysical tuning curves obtained at different test tone frequencies for one monkey is shown in Figure 8, together with the underlying pure tone detection function.

These double-barrelled functions, measuring both frequency selectivity and threshold in the monkey, and intensity discrimination and tone detection threshold in the guinea pig, are distinctly advantageous, for two very different characteristics of the same subject's acoustic behavior may be evaluated simultaneously under normal conditions and following exposure to a toxic substance. Recent findings (*7*) indicate that frequency selectivity as measured by psychophysical tuning curves in chinchillas treated with kanamycin is unaffected at acoustic frequencies where the loss in threshold sensitivity is as great as 50 dB. Greater shifts in threshold were correlated with severely distorted tuning curves. Related histopathological findings from the inner ears of these animals suggest that when outer hair cells are missing from the cochlea no changes are observed in frequency selectivity; however, when inner hair cells in addition to outer hair cells are destroyed, impairment of frequency selectivity follows; psychophysical tuning curves are drastically altered.

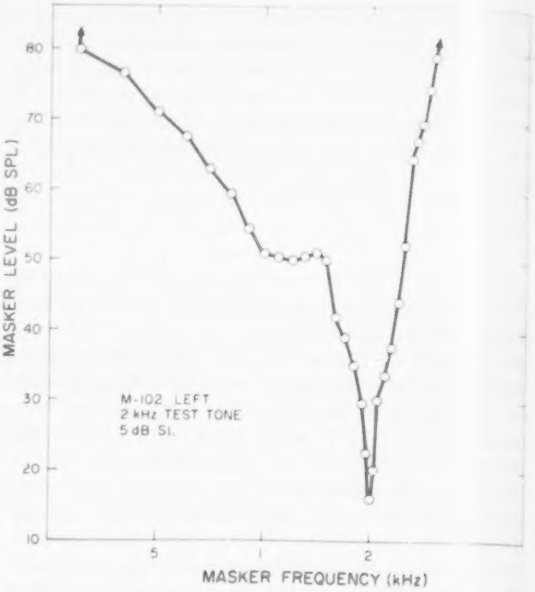

FIGURE 7. Psychophysical tuning curve for a macaque monkey (1).

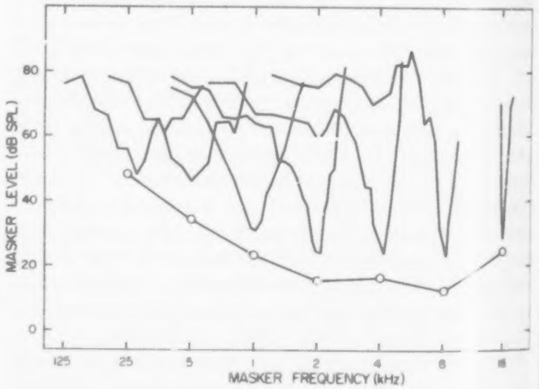

FIGURE 8. A family of psychophysical tuning curves shown with underlying pure tone threshold function for a macaque monkey.

Certain toxic substances, such as the aminoglycoside antibiotics, are highly specific in their action, which is confined to only one or two target sites such as the auditory periphery and the kidney. The behavioral measures just described reveal this form of specificity. Other substances—for example, those that affect the nervous system—appear to produce considerably more diffuse effects that are reflected in a broad spectrum of behavioral changes, often difficult to evaluate in a rigorous, quantitative manner. Some acoustic behaviors are under more central nervous system control and may be severely altered by auditory system changes which are not simply confined to the inner ear. The accurate localization of sound which requires the two ears depends on the integrity of certain structures in the brain stem and on higher levels in the nervous system. Discrimination of speech and communication sounds and other complex biologically relevant signals also appears to rely more on the integrity of central than peripheral structures. Sites in auditory and adjacent cortical regions have been implicated in both sound localization and in the perception of these complex signals (*8*, *9*). In attempting to understand more fully behavioral changes influenced by toxic substances and their underlying target sites, animal models must be developed for these more complex acoustic behaviors.

A procedure for studying sound localization in monkeys shows promise as a potential baseline for behavioral toxicological investigation (*10*, *11*). The procedure is yet another variant on those already discussed and requires the animal to respond whenever an acoustic signal changes location from a standard reference position in front of the animal. Sound sources are located on an arc partially surrounding the animal so that localization acuity may be measured by reference to the angular distance separating any given source from the reference. For example, with the reference sound source at the end of an imaginary line extending from the center of the animal's head between the two eyes to a position directly in front (in the line of regard or 0 degrees azimuth) and a second source at the end of a similar length line extending from the center of the head through one ear, the calculated angle would be 90°.

The preparation is pictured in Figure 9. The animal responds to a light, indicating the beginning of a trial, by closing a switch, and a series of sound pulses is produced from the reference sound source at zero degrees azimuth. In time, the sound shifts briefly to another location on the arc and then returns to the reference position. If the subject breaks contact by removing its hand from the switch when the sound source changes position, food reinforcement is given immediately. On subsequent trials the position to which the sound shifts is varied; acuity of sound localization is taken as the minimum audible angle—the angle in degrees between the reference and the new sound source location on the arc to which the subject responds correctly on 50% of the trials.

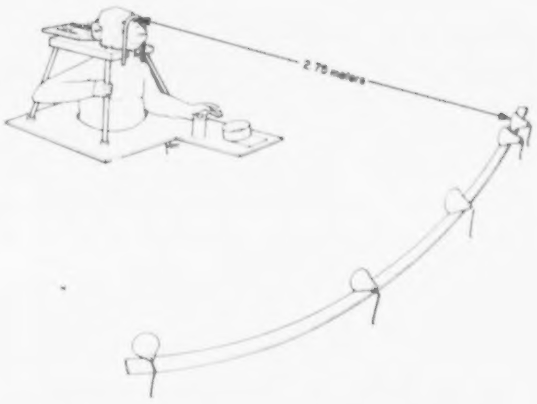

FIGURE 9. Schematic representation of a monkey in restraining chair. Response disk and feeder cup are shown together with arc containing loudspeakers. Reference speaker is indicated by the straight line extending from the center of the animal's head (*11*).

In Figure 10 the percentage of correct responses is plotted for three monkeys as a function of the angular separation in degrees of arc between the reference location and the other positions on the arc to which the sound was shifted. In this particular instance the acoustic signal was a pure tone at 8000 Hz. The acuity of sound localization as measured by the minimum audible angle was about 10° for the three animals. The small number of responses at zero degrees reflected the animals' guessing rate when the position of the source had not changed. It is well known that experimental lesions in the auditory portion of the nervous system will produce severe deficits in sound localization ability (*12*); however, the use of the sound localization paradigm in behavioral toxicology has not yet been entertained.

The processing and perception of complex biologically salient acoustic signals like sound localization is a form of acoustic behavior which is of critical importance in the daily life of man and other mammalian vertebrates. Like localization this behavior shows evidence for central as opposed to peripheral nervous control. Animal models for the analysis of this form of behavior in the laboratory have been and are currently being developed. We know that some animals, other than man, have

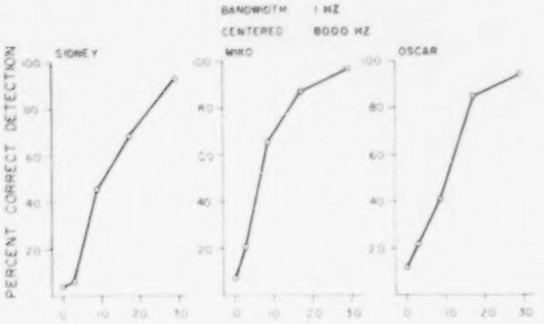

FIGURE 10. Percent correct detection of a change in the location of an 8000 Hz tone as a function of the horizontal displacement of the tone in degrees of arc for three macaque monkeys (*10*).

little difficulty discriminating between, for example, basic human consonant-vowel pairs (phonemes) such as "ba" and "da" (*13*) and between certain variations in their own communication sounds (*14*). The experiments apply operant conditioning principles and represent slight modifications of those procedures described earlier.

An important property of human speech and many animal communication sounds and one that clearly conveys information regarding message content and perhaps also speaker identity is the slow or quite rapid frequency changes that occur so often in the communication process. As with sound localization, there is compelling evidence that perception of these frequency shifts is mediated by the central nervous system (*15, 16*). In our laboratory, we have isolated the frequency shift from communication, speech, or other complex acoustic signals and have presented it to monkeys and guinea pigs as a simple pure tone time varying in frequency. The signal is actually synthesized by computer. The animals are asked to discriminate the frequency-modulated (FM) tone from a simple steady-state pure tone. The FM tone can be varied in rate and extent of modulation (that is, how fast the frequency shift occurs and how far it sweeps across the frequency spectrum). Threshold for FM detection represents that modulation to which the subject responds on half of the trials. Several experiments are planned for this particular preparation. In one series we hope to replicate some interesting findings for human subjects which indicate that exposure (or adaptation) to a frequency-modulated tone can markedly increase the FM detection threshold, thus suggesting the possibility of either channels or perhaps even feature extractors in the nervous system for FM (*15, 17, 18*). Subsequently, the FM detection baseline is one which should be tested for its sensitivity as a baseline for behavioral toxicology.

I am not familiar with any epidemiological evidence from man suggesting that such complex acoustic behaviors as sound localization or the perception of complex biologically relevant signals are adversely affected by exposure to particular toxic substances. Yet, to my knowledge, the specific questions have not been asked. The effects we hope to pick up are sufficiently small that it is somewhat unlikely that they would have been observed earlier without employing the kinds of procedures that we have described here.

The purpose of this paper has been to call attention to a somewhat different strategy for examining the effects of toxic substances on behavior and on the nervous sytem. The procedures suggested are by way of example and are, in no way, intended to limit the possible techniques that might be applied. The basis of the approach argued here is the analysis of behaviors and the elaboration of behavioral baselines that go beyond toxicological screens and detection of simple stimuli. The use of animal models under stimulus conditions which more closely approach those important in real life situations such as perception of communication signals and of their critical elements or the ability to locate the source of a sound in space is advocated in this paper. Perception and discrimination of such complex biologically significant stimulus events is under central nervous system control, and it is well known that many chemical and other poisons exert their toxic effect on the central nervous system (*19*). Perhaps then, the study of experimentally induced perceptual disabilities in these animal models may aid in our understanding of the nature and extent of the disabilities themselves as well as their physiological and morphological basis.

Much of the research described herein was supported by a research grant, NS 15408, and a program grant, NS 785, from NIH.

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Environmental Health Perspectives
Vol. 44, pp. 87-92, 1982

Laboratory Methods of Assessing Hearing Loss

by Aage R. Møller*

Methods for assessing hearing loss in human and in animals are reviewed with special reference to the use of brainstem auditory evoked potentials (BSEP). The technique of recording and digital filtering of BSEP is described and compared with the results obtained by use of traditional analog filtering. The use of electrophysiological methods in assessing threshold shifts in studies of the effect of noise exposure on hearing in experimental animals is described for examples of results obtained in rats.

There are several objective methods available for assessing hearing loss. Some of these methods are suitable for use in studies of the hearing loss induced in experimental animals by noise trauma as well as in man. This presentation will discuss only the recently developed methods that make use of the evoked potentials that can be recorded from various locations in the nervous system.

The first response that can be recorded from the auditory nervous system in response to a transient sound is the compound action potential of the auditory nerve. That potential can be recorded either from the round window of the cochlea or from the auditory nerve where it leaves the ear and enters the brain through the internal auditory meatus. The other nuclei of the ascending auditory pathway also generate electrical potentials in response to a sound. Due to neural delay, these potentials appear at a later time than does the response from the auditory nerve. However, a characteristic common to all of these responses is that they can only be recorded in response to changes in sound and they are more pronounced when the sound stimulus is a transient sound than a sound of slowly varying intensity. Figure 1 shows an example of a recording of the compound action potential made from the round window of the cochlea in an anesthetized rat. The response is characterized by two peaks, the first of which appears about 1 msec after the onset of the tone burst that was used as the stimulus. This N_1 potential is assumed to reflect the electrical activity in the auditory nerve that results from stimulating the ear with a transient sound. The second peak (N_2) appears about 1 msec later, and it is believed to originate in the first relay nucleus of the ascending auditory pathway, namely the cochlear nucleus. The amplitude of the N_1N_2 potential decreases when the sound intensity is decreased and the latency increases. These potentials exhibit a threshold, which means that below a certain stimulus intensity they can no longer be recorded.

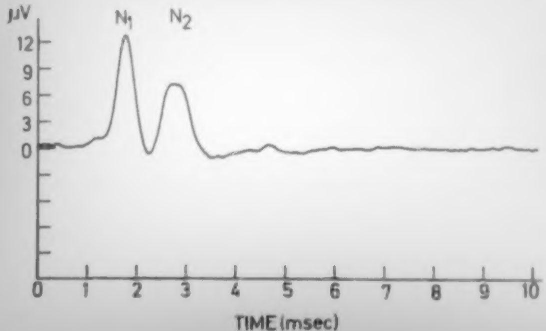

FIGURE 1. Typical compound action potential recorded from the round window of the cochlea in a rat. The stimulus was a brief click delivered to the ear via an earphone. The time scale gives the time in milliseconds after the occurrence of the click.

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When these potentials are recorded directly from the nerve or the nuclei in response to stimuli well above threshold, they have amplitudes of many microvolts and can, therefore, be directly visualized on an oscilloscope when the sound stimulus is well above the threshold of hearing.

Near threshold, the amplitude of these compound action potentials becomes smaller than that of the background noise (originating from other biological or electrical activity in the animal being tested and from the amplifiers). It therefore becomes necessary to present the same stimulus many times and average the responses to the stimuli in order to be able to identify these potentials. Such average techniques are in common use both in the laboratory and in the clinic. The averaging procedure increases the signal to noise ratio by a factor of $\sqrt{N}$, where N is the number of responses averaged. The responses can be averaged using specialized (usually digital) equipment or using a minicomputer.

Recordings of the compound action potentials have been used in animal experiments to study the effect of noise exposure on the ear. For this application, the sound levels, at which the compound action potentials were just discernable after averaging 500 to 1000 responses, were determined. These threshold sound levels were determined when bandpass-filtered clicks were used as stimuli. Bandpass-filtered clicks are generated by passing a short rectangular pulse (usually of a duration of 10 to 50 μsec) through a suitable bandpass filter. The output of the filter is a damped oscillation with a frequency equal to the center frequency of the bandpass filter. The center frequency of the bandpass filter is varied over the range of frequencies where the threshold is sought. An example of such use of the threshold of the compound action potential is shown in Figure 2, which presents the thresholds of the compound action potentials in rats exposed to noise of the same energy but distributed differently over time.

These experimental results shown in Figure 2 were obtained in a study designed to test the so-called equal energy hypothesis of noise damage. This hypothesis states that it is the total noise energy to which ears are exposed that determines the degree of hearing loss. The different curves in Figure 2 show the thresholds for different stimulus intensities when the total exposure time was adjusted so that the total energy of the noise was the same. The noise was a one-octave band centered at 8 kHz. The bandpass-filtered clicks used as test sounds to determine the thresholds were generated by passing 50-μsec rectangular pulses through a 1/3 octave filter (Bruel and Kjaer). It may be seen that the rats which were exposed to the lower noise intensities suffered hearing losses of about the same magnitude, thus supporting the equal energy hypothesis of noise damage. However, it is also evident that exposure for a short time to a high intensity noise is more damaging than exposure for a longer time to a less intense noise. These experiments are examples of ways in which electrophysiological methods may be used to assess the damaging effect of noise on the inner ear. These results may not be exactly identical to those obtained by behavioral evaluation of hearing loss, but they are valid for the purpose of comparison between normal (nonexposed) animals and noise-exposed animals. The electrophysiological method is frequently preferable to the behavioral method of determining hearing loss because it is much faster and the results are more readily reproducible.

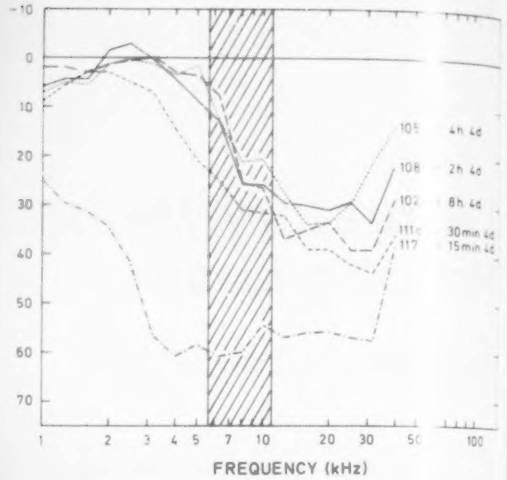

FIGURE 2. Threshold shift for the compound action potentials recorded from the round windows of rats that were exposed to noises of different intensities. The duration of the exposure was so adjusted that the total noise energy was the same. The curves represent the mean value of the results obtained in five rats for each type of noise exposure. The test stimuli were bandpass-filtered clicks (1/3 octave bandpass filters were used) and the noise used to induce the hearing damage was bandpass-filtered white noise one octave wide, centered at 8 kHz.

Recordings from other parts of the ascending auditory pathway may also serve the purpose of determining hearing loss in experimental animals. In addition, such recordings may give other information about the effects of trauma on the auditory nervous system. For instance, it is possible to discern an effect on the ear from exposure to various chemicals.

When a recording electrode can be placed close to

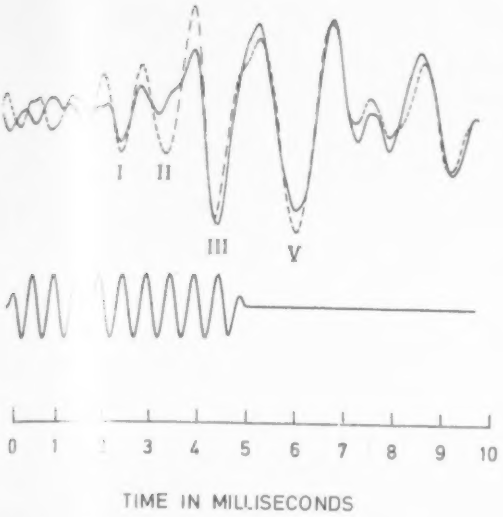

FIGURE 3. Typical response to tone bursts recorded in a human subject with normal hearing. The recording electrodes were placed on the mastoid and the forehead. The sound stimuli were 2000-Hz tone bursts, delivered to the ear from the mastoid of which the response was recorded. The tone bursts had a duration of 5 msec and were presented with an interstimulus interval of 150 msec. The hearing level of the tones was 75 dB. Two thousand responses were averaged. From Møller et al. (*2*).

the nerve or nucleus from which the recording is made, the potentials are relatively large and the number of responses that need to be averaged is relatively small. When recordings are done in man, the situation is usually different. Auditory evoked potentials in man can be recorded from the round window of the cochlea, but that is an invasive procedure and therefore used only in special cases. The most common method for recording auditory evoked potentials in man is to record from the scalp. Recorded in that way, the response to a transient sound such as a tone burst or a click sound is characterized by a series of peaks. During the first 10 msec after the onset of a sound, the so-called brainstem evoked potentials can be seen. When one electrode is placed on the mastoid and one on the vertex or forehead, the potentials recorded consist of a series of six to seven peaks (Fig. 3). The mastoid negative peaks are usually given Roman numerals from I to VI. Due to the large distance between the neural generators and the recording site, the amplitudes of the auditory evoked potentials recorded in that way are much smaller than those of the background noise (in this case the person's spontaneous brain activity and the noise of the amplifier, the latter being of a much smaller amplitude than the former). It therefore becomes necessary to average a large number of responses (usually several thousand) in order to be able to identify the reponse. Since the ratio between the signal and the noise only increases with the square root of the number of responses averaged, the reduction of noise is a slow process when the signal is small and the background noise is large. It is, therefore, important to use other methods to reduce the background noise. One such method is spectral filtering. If some of the energy of the response is located in different frequency regions from that of the background noise, it is possible to remove some of the background noise by spectral filtering. This technique is now widely used, but unfortunately spectral filters also introduce distortion of the waveform of the recorded potentials. The filters used presently are mostly of the common analog type that attenuate either high frequency energy (lowpass filters) or low frequency energy (highpass filters). Usually both types of filtering are used and the signal is usually led through these filters before it is fed into the averager.

The distortion of the waveform that analog filters introduce by the unavoidable phase shift of such filters limits the extent to which such filters can be used to reduce noise. The availability of digital computers has made it possible to filter signals in ways that are not possible using analog filters. The design of such digital filters is much more flexible than that of analog filters since they do not need to be physically realizable (*1*). Figure 4 illustrates that it is possible to design a filter that does not distort the waveform of a typical evoked potential recorded from the scalp of a human subject. Particularly it may be noted that the latencies of the peaks are unchanged by filtering. These digital filters, thus, do not introduce distortion of the features that are of interest. Since it is the location of the peaks in time that is interesting, it is advantageous to enhance the peaks by using a suitable filter; this can be done easily, as seen from the recordings shown in Figure 4.

Analog high- and lowpass filters, which have about the same cutoff frequencies as the digital filters, introduce significant distortion of the recorded pattern, as can be seen from Figure 5. Of particular importance is the fact that the analog filters shift the location of the peaks in time, which the digital filters do not. Since the latencies of the peaks often are the important characteristic of these potentials, such distortion cannot be tolerated. Therefore, when analog filters are used it is necessary to select filters with a wider passband and thus, those which are less efficient in reducing the background noise.

The efficiency of the filter in enhancing the

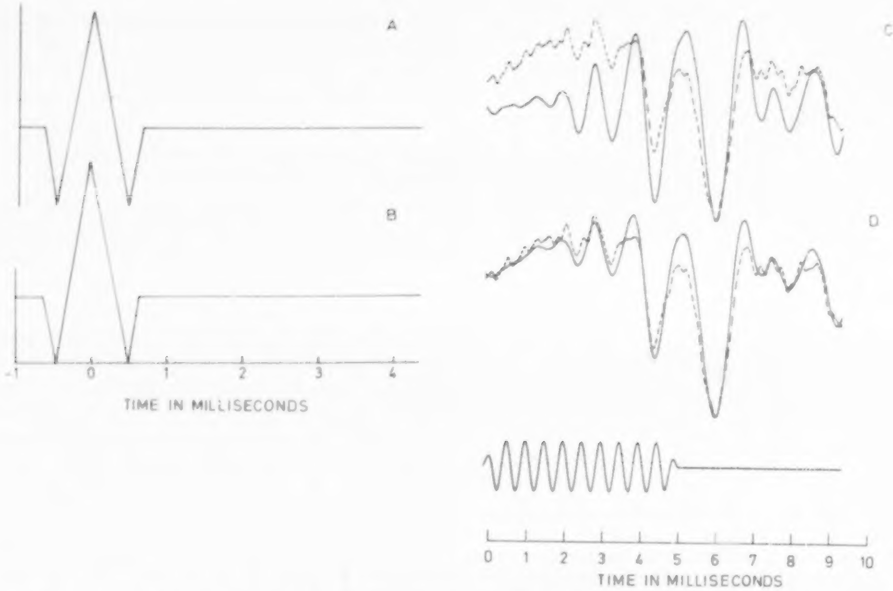

FIGURE 4. Illustration of the distortion introduced by two different digital filters (A and B) together with the weighting function of these two filters. The dashed line is the original (unfiltered) response, and the solid lines show the filtered response. The responses may be seen as representing a "clean" record of the brainstem evoked potentials since they are the average of 20,000 responses obtained in a person with normal hearing in response to 2000-Hz tone bursts presented at 75 dB above the normal threshold of hearing.

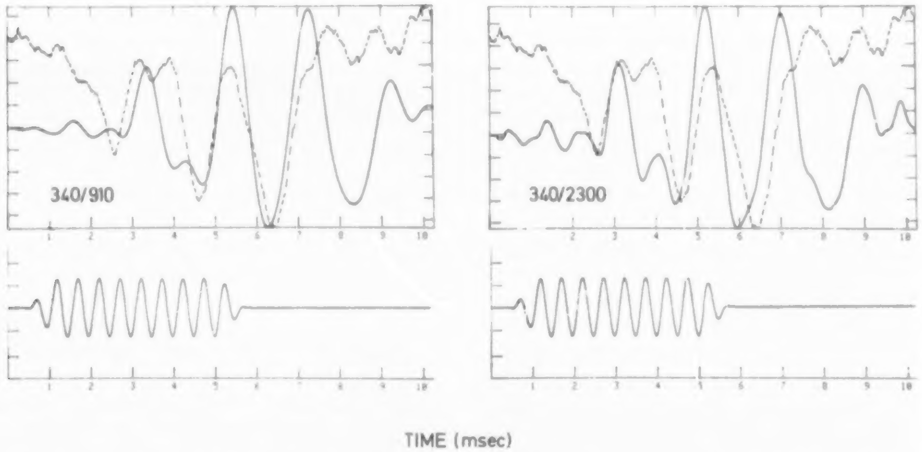

FIGURE 5. The same responses as shown in Fig. 4 but filtered by analog filters. The dashed lines are the unfiltered responses and the solid lines are the filtered responses. The filters were 18 dB/octave, minimum overshoot analog high- and lowpass filters. The cutoff frequencies were different for the two graphs and are shown by legend numbers.

response over the background noise is different for analog and digital filters. This is illustrated in Figure 6, which shows the effect of the two types of filters on the recorded scalp potentials in an individual when only a few (128) responses were averaged. It is seen that the responses comprising the different segments of the averaged responses are more similar when digital filters are used than when analog filters are used. That indicates that the digital filters shown in Figure 3 are more

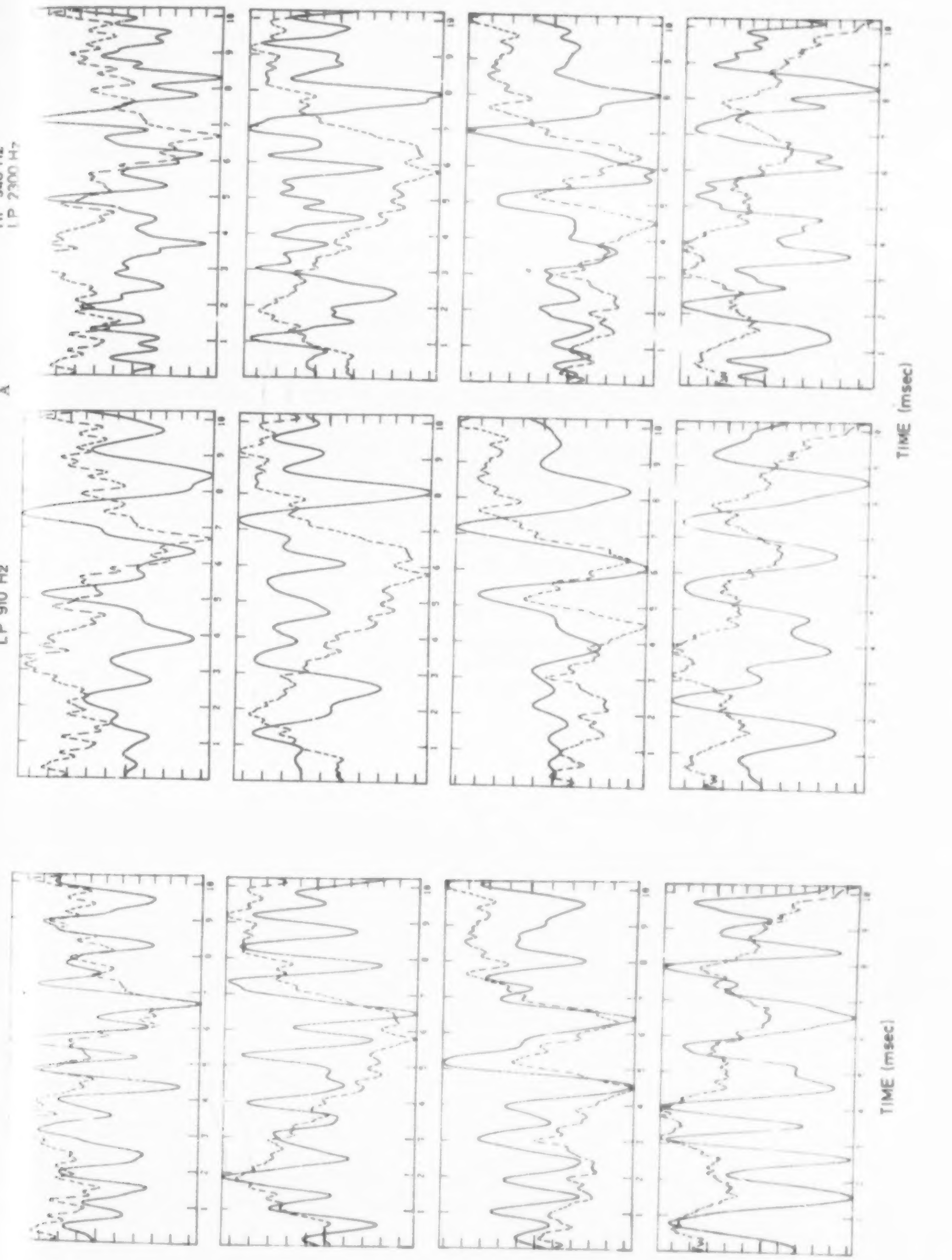

FIGURE 6. omparison of the effects of (A) digital and (B) analog filters in enhancing the responses in the presence of background noise w only a few responses are averaged. The cutoff frequencies of the analog filters were the same as shown in Fig. 5 (and given b gend numbers) and the digital filters were those described in Fig. 4.

efficient than are analog filters in enhancing the response in the presence of background noise. The digital filter shown in Figure 3 has been in practical use in the Division of Audiology at the Eye and Ear Hospital for about two years (*2*).

The evoked potentials recorded from the scalp can be used in assessing the degree of hearing loss either by determining the threshold of these potentials or by comparing the sound intensity required to obtain a certain amplitude of the evoked potentials. The latencies of the various peaks in the scalp-recorded brainstem evoked potentials (BSEP) decreases with increasing stimulus intensity. This can be made use of in determining the loss of hearing sensitivity by determining the sound intensity required to obtain a response with a certain latency. In the impaired ear, a higher sound intensity is required compared to the normal ear and the difference between these two sound intensities is a measure of the loss of sensitivity of the ear. However, this method is not suitable when the damage to the ear is located in the sensory epithelium of the inner ear. In that case the so-called recruitment of loudness makes the response of an impaired ear to sound above threshold almost equal to that of an unimpaired ear. The fifth wave of the scalp-recorded BSEP (see Fig. 3) is often used to determine the hearing threshold in a person who for one reason or another cannot cooperate sufficiently to undergo ordinary pure tone audiometry. This method of recording BSEP is also suitable for assessing the hearing loss present in experimental animals or for assessing the degree of damage to the hearing caused by noise exposure, etc. The pattern of the BSEP is different from animal to animal and between animals and man. Determination of the threshold in man is done most easily by observing the fifth peak. In animals, it may be the fourth peak that is most easy to observe to obtain the threshold. The BSEP can be recorded from the scalp in awake or anesthetized animals using surface electrodes or needle electrodes. Such measurements can be repeated in the same animal as many times as required.

When the purpose of the study is to compare the threshold in normal animals with that of animals with hearing losses, it is of no concern that the absolute value of the threshold obtained using the electrophysiological method is not exactly identical to that obtained by behavioral methods. As long as the same reocording technique is used for the normal and the experimental animals, the BSEP method gives an accurate measure of hearing loss.

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Environmental Health Perspectives
Vol. 44, pp. 93-100, 1982

Psychophysical Methods for the Measurement of Somatosensory Dysfunction in Laboratory Animals

by Patrick A. Cabe*

Somatosensory dysfunction is a widely reported clinical consequence of chemical exposure. Assessment of such dysfunction should be an important component of agent safety testing, necessarily implying evaluation of psychophysical functions in laboratory animals. The logic of testing agent-induced sensory dysfunction, conceptual and practical factors affecting such tests, and the categories of experimental methods available are reviewed.

Introduction

As Arezzo (*1*) has noted, the great length of the peripheral nerve fibers makes them exquisitely sensitive to neurotoxicants. Motor function mediated by the long fibers innervating the hind limbs is frequently affected early in cases of neurotoxicosis (*2*).

The peripheral nerve trunks carry both motor and sensory impulses, and sensory dysfunctions also are widely reported neurotoxic signs. Maurissen (*3*) has tabulated the array of agents, both drugs and toxicants, known to yield somatosensory dysfunction. Decreased vibration sensitivity, altered temperature sense, impaired tactile ability, numbness, and a variety of subjective effects have been seen clinically.

Clinical effects are human health consequences of agent exposure, surely a crucial component of the total environmental health research effort. More important, however, must be the prediction of human health risks of agent exposure and the elucidation of mechanisms of injury (*4*). We want to know what effects may occur in exposed human subjects, and by what means such effects occur, without in fact exposing human subjects to potentially dangerous agents.

The general objective, then, is to demonstrate agent effects in nonhuman models, in the laboratory under controlled conditions of maintenance and exposure. Because the somatosensory systems are affected early by a variety of agents, assessment of somatosensory function requires a high priority.

I will review aspects of the broad problem of testing nonhuman subjects for agent-induced somatosensory dysfunction, discussing categories of somatosensory functioning; requirements for psychophysical assessment of sensory function; the logic of inferring agent effects on sensory functioning; and several general procedures for animal psychophysical testing.

Categories of Somatosensory Function

Any major textbook or reference source can be consulted for an overview of somatosensory function (*5–8*). A convenient summary groups functions by anatomical locus (Table 1). Within each grouping, subgroupings of phenomenal or subjective experience can be identified. Thus, the cutaneous senses include touch-pressure, temperature, vibration, and superficial pain.

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Table 1. Categories of somatosensory function.

Sense	
Skin senses	Touch-pressure Temperature Vibration Superficial pain
Deep senses	Muscle, joint, tendon sensation Deep pressure Deep pain
Visceral senses	Organic sensation (hunger, thirst, nausea) Visceral pain

Cutaneous sensitivity has probably been most extensively studied, because the locus to be stimulated is easily accessible. What follows here is oriented toward the measurement of cutaneous sensitivity exclusive of pain. [The psychophysics of pain is discussed elsewhere by Weiss and Laties (*9*).]

Despite the ease of access to the body surface, tests of cutaneous sensitivity are complicated by a range of factors beyond the magnitudes of physical stimulation applied. A simple example is the variability in sensitivity to two-point stimulation as a function of locus of stimulation (*8*): the tongue, for instance, is very sensitive, the back and thigh much less so.

Other factors modulate the phenomenal experience of cutaneous stimulation. Spatial patterning is implied directly in the measurement of two-point thresholds: two points of contact are experienced as one if the two points are sufficiently close together. Simultaneous spatially distinct contacts may have inhibitory, summatory, or other effects (*7*).

Temporal interactions also occur. Adaptation to prior or co-occurring stimulation (for example, different temperatures) may alter the subjective magnitude of stimulation.

Temporal and spatial patterning may interact. A tactile phi phenomenon (*7*), in which cyclic stimulation of two spatially distinct loci yields the experience of a single contact moving between the two sites, is well known. Neurologists take advantage of spatial-temporal patterning in tests of recognition of letters drawn on the skin and of objects by touch (*8*).

Intermodal effects among the cutaneous senses yield higher order experiences, as in "touch blends" (*5*). The experience of "oiliness," for instance, can be reduced to weak pressure plus warmth. The matching of objects examined tactually to samples presented visually is well known, even in nonhuman primates (*10*).

Kennedy (*11*), in a further interesting example of higher order information available to skin senses, has reported that congenitally blind subjects are capable of identifying common objects depicted as raised ridges (the tactile equivalent of lines). Some blind subjects, asked to draw common objects, spontaneously invented line depiction conventions of interposition and linear perspective.

In summary, the experience of the environment available to the cutaneous receptors can occur on many levels, from those effects rather easily referred to the periphery to others which have complex cognitive components. Such effects pose challenging problems, and, in principle, any and all could be affected by exposure to toxic agents.

Characteristics of Psychophysical Assessments

In all the examples cited above, the issues are psychophysical ones. The general psychophysical problem is to discover the relation between some varying character of physical stimulation and variations in the experience of such stimulation. Physical stimulation is often multivariate (e.g., vibration varies in frequency and amplitude), so psychophysical assessments often yield families of functions.

Every psychophysical experiment involves three components. First, the characteristics of the physical stimulation must be carefully controlled. Control of stimulation requires attention to details of stimulus generation, calibration of stimulus-generating devices, and, often, monitoring of stimulation parameters during presentation. Second, the locus of stimulation must be specified, both the specific body part and the extent and/or shape of the area stimulated. Locus is particularly important when repeated tests of function are intended, as across days, weeks, or months. Third, a means by which the subject can report his/her experience of the stimulation must be provided. Reports can range from very simple (a switch closure or a yes-no verbal report) to very complex (an introspective description).

Procedural details go beyond these three major components. The order of presentation of stimuli, their context (e.g., background or adaptation stimulation), and the kinds of comparisons to be reported all affect the design of particular investigations. Engen (*12*) provides a concise survey of these issues.

All three of the major components listed pose difficulties for psychophysical assessment in animals. Perhaps the biggest hurdle is the requirement for a response mode that can vary with changes in stimulation. At best, one can get a crude categorical response (on the order of low-medium-high, or yes-no-not sure) from the animal, and most frequently investigators settle for some form of a yes-no response. Even so, guaranteeing that the response is being made appropriately, to the aspect of stimulation of interest and not to extraneous cues (e.g., apparatus noises) may require extensive preliminary training and/or a variety of control tests (13). The training investment, on the other hand, may purchase exquisite precision in the control of responding by the stimuli of interest.

Control of the locus of stimulation with human subjects is a relatively minor difficulty because we can enlist the subject's cooperation via verbal instructions. The animal subject either must be trained to maintain an orientation toward stimuli, be restrained in some manner, or both, in order that stimulation can be reliably applied to a specifiable body locus.

Always, precisely describable stimuli must be generated. The emergent problem here is that, in animal psychophysics, automation of the stimulus presentations, their order and variations, is desirable. The additional equipment required may increase the cost and complexity of animal psychophysical study.

Assessment of Agent Effects on Sensory Systems

In general, a sensory dysfunction can be inferred from the observation that the psychophysical function for an agent-exposed subject is displaced relative to, or of a different shape than, the corresponding function in a nonexposed subject. Such evaluations are multivariate: while response change (or some derived measure, such as probability of response) is the single dependent variable, independent variables may include the parameters of the stimulus of interest (e.g., frequency and amplitude), yielding a family of curves, and must include a range of doses of the agent in question. Results reviewed by Stebbins and Moody (*14*) illustrate these points.

Since we infer altered sensory function from altered response, it is critical that nonsensory explanations for altered response be ruled out. Peripheral neuropathies, as a case in point, yield motor as well as sensory signs. Since some psychophysical procedures used with animals manipulate motivational levels (via food or water deprivation), and since ingestional changes often occur in toxicosis, motivational changes must be considered as a possible factor in response alterations.

Finally, modern psychophysical thinking invokes the concept of response bias, referred to an internal criterion for reporting detection of stimulation. Estimation of response bias is a central aim of signal detection theory (*15*). Signal detection methodology has been extended to the analysis of animal psychophysical studies in a number of cases. In principle, an effect of toxic agents may be to shift the subject's response criterion, an effect that would be reflected as a change in response bias, while detectability of the stimulus may or may not be affected.

Overview of Animal Psychophysical Techniques

A variety of sources on animal psychophysical methods are available, with extensive bibliographies (*13*, *16*, *17*). My aim here is to critically review some of the available techniques for their usefulness in assessing agent-induced sensory dysfunction. The principal context is the evaluation of somatosensory function, but it must be stressed, on the one hand, that there are very few apparent applications of any of these methods in the somatosensory domain and, on the other hand, that any of these methods could probably be applied to any sensory modality, given some ingenuity. Restrictions on applications are logistical and engineering problems rather than conceptual ones.

The overview presented here is summarized in Table 2, where candidate procedures are rated against major considerations in choosing a technique.

Factors in Evaluating Animal Psychophysical Methods

Factors Related to the Psychophysical Function

There are three such factors: control of the parameters of the physical stimulation; control of the body locus, or sensory organ, stimulated; and the precision of the psychophysical function(s) that can be obtained. The first two have already been discussed. The precision of the psychophysical function refers to the degree of variability resulting from any of a number of sources, perhaps the greatest of which is the "tightness" of the association between stimulus and response. Some methods are intrinsically more variable; others may provide for exquisite control of responding by stimulation.

Table 2. Comparison of animal psychophysical procedures.

Factors affecting choice	Method[a,b] 1	2	3	4	5	6	7
Control of stimulation parameters	0	+	0	–	+	+	+
Control of locus of stimulation	0	+	0	–	+	+	+
Precision of psychophysical function	–	+	0	–	+	+	+
Sensory-motor separation	–	0	+	–	+	0	0
Possibility of, need for response bias measurement	+	+	+	–	+	0	0
Motivational manipulation required	+	+	+	+	+	–	–
Amount of response training required	+	+	+	+	+	–	–
Amount of testing time required per function	–	+	–	–	+	0	–
Repeatability across time	+	+	+	+	+	+	+
Applicability to neonates	+	+	+	–	+	0	0
Labor intensiveness	–	0	0	0	0	+	+
Automatability	–	+	0	0	+	+	+
Equipment cost, complexity	+	0	0	+	–	–	–

[a] Methods are rated as follows: (+) advantage; (–) disadvantage; (0) no clear advantage or disadvantage.

[b] Methods are as follows: (1) reflex, orientation, or forced movement (kineses) measures; (2) reflex modulation; (3) habituation/dishabituation; (4) preference, tropism measures; (5) classical conditioning; (6) operant conditioning; (7) conditioned suppression.

Factors Related to Alternative Explanations for Altered Psychophysical Functions

Three factors (separation of sensory from motor components; response bias; and altered motivational milieu) have been discussed above. Psychophysical methods differ with respect to the degree that sensory and motor components of observed response change can be controlled or measured or come into play. Some methods, for instance, may utilize autonomic response: thus, the motor component is negligible. In other cases, responses to varying stimuli may be evaluated as relative to one another; altered motor components, if they are homogeneous across the stimulus range being tested, may be taken out of consideration.

Response bias in some sense is an analytical entity. That is, under appropriate experimental designs, it can be evaluated separately from detectability. Blough (*18*) provides an example of how such analyses can be performed. A range of designs lend themselves to response bias evaluation and the possibility of doing so is of some advantage.

Factors Related to Logistics and Design of Experiments

A prime motivation for listing this set of factors is that a large number of agents stand in need of some testing (*19*). Consequently, the time required to train and test subjects is an important consideration. Since dosing regimes that simulate environmental exposures are typically chronic and since the effects may be expected to be cumulative, methods that are repeatable across time (within-subjects designs) are most useful. Given the continuing strong interest in the evaluation of pre- and perinatal exposures, methods applicable (at least in principle) with very young organisms would be especially useful.

Economic Factors

No laboratory has infinite resources. Therefore, cost factors inherent in any method must be taken into account. The labor intensiveness and the level of such labor (e.g., kind and amount of training required) affect costs. Some methods may be automated, at some cost for development and for equipment, balanced off against labor costs saved, precision obtainable, and the possibility of testing more animals per unit of labor.

Candidate Procedures in Animal Psychophysics

Reflex, Orientation, and Forced Movement (Kineses) Measures

Reflexes are "prewired" motor or autonomic responses to stimuli, usually of a limited sort applied to a limited body region; the neurology literature provides numerous examples. Orientation measures refer to movements directed toward stimuli; these may be at a distance (sound, light, or chemical source) or on the body surface (a tactile stimulus applied to the skin). Kineses (*20*) refer to increases in activity as a function of imposed stimulation, e.g., increased movement at higher temperatures. Rough psychophysical functions can be derived by observing the variations in probability, amplitude, and/or latency of any such response as stimulus parameters are changed (*19*, *21*).

The major advantages of such methods are that, since the responses are built in, no training or motivational manipulations are necessary, and response bias is not a problem. Tests can be repeated in the same animals across time, so long as short-term effects (habituation) are allowed for. In principle, where appropriate reflexes can be identified, these methods should be applicable to neonatal animals. Equipment costs will probably be low.

The principal disadvantage is that, since reflexes are by definition sensory-motor arcs, sensory components may not be separated easily from motor effects. The functions obtained may be relatively imprecise, and the amount of hand labor required to derive functions may be quite large. These procedures are not readily automated.

Reflex Modulation

Hoffman and Ison (*22*) point out that the magnitude of a reflex (for instance, a startle response to a sharp noise) can be depressed by precedent or co-occurring stimuli, and that the reflex inhibition is related to the detectability of the precedent stimulus. This prepulse inhibition of reflexive response obviously can be incorporated into a psychophysical experiment. Russo (*23*) has reported the successful use of this technique to detect sensory dysfunction induced by environmental agents.

Reflex modulation holds great promise as a method for assessing agent-associated sensory dysfunction. Control of stimulation can be excellent, both in location and parametrically. The functions obtained (*23*) can be quite precise. Because the method is reflexive, response bias is not a problem and no motivational manipulation is required. Also, no special response training is necessary and the evaluation can be repeated across time. The method is useful with neonates (*24*) and can be automated, in at least some applications.

Habituation/Dishabituation

Presentation of a novel stimulus frequently can be seen to produce an orienting response, with both motor (startle) and autonomic (e.g., heart rate change) components (*25*). With repeated presentations, the orienting response wanes (habituation). Presentation of a second stimulus following habituation to the first may reinstate the orienting response (dishabituation). The habituation/dishabituation paradigm may be used as a psychophysical method.

An example of this approach is the work of Moffitt (*26*), who found that very young human infants could discriminate small speech sound differences. Infants showed habituation of a heart rate change to the syllable "ba" and dishabituation to a second syllable, "ga", which differed in one phoneme from the first.

Habituation/dishabituation has the advantage of potential for separating sensory from motor effects of toxicants, in that autonomic measures can be used. This supports the method's use with neonates, too, where motor control may not be well developed. In fact, the method might be usable with some organisms prenatally. As with other reflex measures, response bias is not likely to compromise inferences of sensory dysfunction.

The major drawback is the amount of time likely to be required to derive a psychophysical function. Since the method employs (multiple) paired comparisons, testing may be quite time consuming.

Note that failure to observe dishabituation does not allow the inference of non-discriminability of stimuli. Observation of dishabituation, however, is clear evidence for discriminability.

Preferences and Tropisms

Most organisms have multimodal preferences for a sensory context and, given the opportunity, will spend more of their time in the preferred context. For some organisms and some stimuli, the movement toward or away from the source of stimulation (e.g., heat, a chemical source) is highly reliable and predictable on the basis of knowledge about the gradient of stimulation present (*20*).

A typical preference experiment employs a choice box of some sort in which stimulus characteristics differ in two regions. By recording the relative proportion of time spent in the two areas, a rough psychophysical function can be generated.

In approximately the same fashion, tropisms may be tested: in this case, a gradient is constructed. For instance, a metal plate with one hot and one cold end specifies a temperature gradient. By noting the proportion of time spent in various zones of the gradient, again, a rough psychophysical function can be plotted.

These approaches have the advantage of relative simplicity. It is possible to automate them, for example, by using photocell arrays along a runway. Equipment cost and complexity generally will be low. Because the procedures depend on built-in preferences, no motivational manipulations are necessary and no special training is required. The method should be repeatable across time, too.

Control of the locus of stimulation will be difficult because the animal controls its own stimulation. The amount of time required to determine a psychophysical function may be protracted. The methods may be labor intensive, but the observations probably do not require a high degree of technical skill.

Since locomotor responses are typically employed, sensory and motor components will probably be confounded. Response bias (vs. detectability) is not obviously measurable. As with habituation/dishabituation, absence of preference does not necessarily imply absence of discriminability.

Classical Conditioning

Classical conditioning can be used effectively in psychophysical experiments. The general procedure is to pair a signal, called the conditioned stimulus (CS), with an unconditioned stimulus (UCS), often mild electric shock. Usually the CS precedes or overlaps the UCS. The UCS elicits an unconditioned response or reflex (UCR), which may be overt, as a limb flexion, or covert, as a heart rate change. After some number of CS-UCS pairings, the CS alone comes to elicit a response like that elicited by the UCS. The response to the CS is called a conditioned response or reflex (CR). The ability of a stimulus to become a CS$^-$ is prima facie evidence for its detectability.

Discriminative responses can also be established, in which case one stimulus is a CS$^+$, paired with the UCS, and another is a CS$^-$, paired with the absence of the UCS. By manipulating the difference between CS$^+$ and CS$^-$ pairs, their discriminability can be tested. Thus, measurement of thresholds for stimulus differences is possible.

Examples of classical conditioning in broadly psychophysical contexts are easily found in the psychology literature of the past century. Some recent applications that illustrate the potential of classical conditioning can be seen in the work of Kreithen and colleagues (*27*). The general problem Kreithen addresses is to discover the possible sensory bases for homing in pigeons.

His procedure is relatively straightforward. Stimuli of interest (the CS) are paired with mild subcutaneous electric shock (UCS) while heart rate is monitored. Heart rate acceleration (UCR) reliably follows shock. If the CS is detectable, it comes to elicit a heart rate acceleration. Conditioning is reported to occur after about 10 CS-UCS pairings; the remainder of a 50-trial session can then be devoted to varying stimulus parameters, during which a reliable psychophysical assessment can be made.

Using this procedure, Kreithen has studied sensitivity to magnetic fields, to barometric pressure changes, to the plane of polarized light, and to very low frequency sound (infrasound).

The only obvious disadvantage in this procedure is that equipment for recording and control of stimulus presentations may be relatively complex and expensive, but classical conditioning promises to be advantageous on most of the other factors listed.

Parenthetically, classical conditioning procedures are major components of Soviet behavioral toxicological assessments (*28*), and are reported to be very effective in demonstrating neurobehavioral toxic effects.

Operant Conditioning

The methodology of operant conditioning in psychophysics is reviewed elsewhere (*12*, *16*, *17*, *29*). Operant procedures have been the most widely applied methods for the precise definition of animal psychophysical functions, for many reasons: The test apparatus can be tailored to the animal under test such that excellent control of stimulus parameters and locus of stimulus application are possible. The timing, location, type, frequency and ultimate control of responding, coupled with the control of stimulation, can lead to extremely precise psychophysical functions. Functions have been shown to be highly repeatable across relatively extended periods of time. Operant methods have developed as highly automated technologies over the years; automation reduces labor costs but generally increases complexity of equipment and equipment costs.

Major disadvantages of operant procedures include the frequent requirement of motivational manipulation (e.g., food deprivation) and the fact that stable responding may only follow very extensive training.

Excellent examples of operant psychophysical methods in the somatosensory domain can be seen in the work of Maurissen (*3*), who is studying detectability of vibratory stimuli in monkeys, and in the approach reported by Burne and T son (*30*). The latter employ a relatively high probability response in restrained rats, a nose poke that is sensed by a photobeam interruption. Step-wise shock level changes are applied to the rat's tail, incrementing on a fixed-time schedule and decrementing contingent on the response. By monitoring the shock level increment-decrement reversals, the rat's threshold for reactivity to electric shock can be tracked.

The presentation of stimulation by itself can be a reinforcing event (*31*). In principle, this could be a foundation for psychophysical evaluation; it resembles the preference method mentioned earlier, with similar advantages and disadvantages.

For some purposes, the precision that can be obtained using operant methods may far outweigh the disadvantages. If, for example, correlations between histopathological changes and functional disruption are the objective (*14*), the results achievable may justify the expense and time. At the other extreme, operant psychophysics may be prohibitively expensive for agent screening ends, at least as such methods are currently known. Given the flexibility operant methods have demonstrated over the years, it may be that ingenius screening applications can be found in the operant psychophysical domain.

Conditioned Suppression

The operant and classical conditioning approaches have been combined in the conditioned suppression method. Animals trained under an operant schedule of reinforcement to emit a stable and moderately high rate of responding are exposed to a signal (CS) which precedes an unconditioned stimulus (UCS), often mild electric shock. Responding is interrupted, suppressed, by the UCS; thus, suppression of response can be considered an unconditioned response. Over some number of CS/UCS pairings, the CS comes to suppress responding. Suppression of responding from a stable baseline in the presence of a CS then is evidence for detectability of the CS.

Typically, suppression is expressed as a ratio between pre-CS responses and CS responses, often calculated so that it can vary between 1.00 (complete suppression) and zero (no suppression). The suppression ratio increases toward 1.00 as conditioning progresses and varies directly with the detectability of stimuli. Applications of conditioned suppression to detectability problems have been reviewed by Smith (*32*) and have been used successfully in a variety of modalities, including cutaneous sensitivity.

Given that conditioned suppression is a hybrid operant-classical conditioning method, it tends to suffer from the disadvantages of both. Particularly, training and testing time, overall, may be excessive, and equipment may be relatively complex and expensive. The operant baseline will generally require a motivational manipulation, though the suppression of responding itself will not.

Since the suppression ratio should be relatively insensitive to baseline shifts from session to session, the psychophysical functions derived should be relatively invariant, except for induced sensory changes

Summary and Conclusions

The investigator interested in assessing agent-induced sensory function changes has a rather broad inventory of methods at his disposal. In general, selection of any psychophysical method entails trade-offs, most frequently perhaps the precision-cost balance, where cost includes time, equipment, and labor expenditures. Simple, manual, quick procedures tend to yield imprecise psychophysical functions; more complex, automated, time-consuming techniques may lead to exquisitely precise functions.

A survey of animal psychophysical studies would demonstrate a preponderance of effort on the visual and auditory systems. Other modalities, from the familiar (taste, olfaction) to the exotic (barometric pressure, magnetic field sensitivity, sensitivity to electrical fields) have also been examined from time to time. The facile generalization is that it is easy to think in terms of the familiar modalities; those that are of less apparent immediacy to human life inspire less interest. However, where the interest exists, it seems that available psychophysical procedures (with some ingenuity) can be brought to bear.

The somatosensory domain is one of those in which animal psychophysical study has been relatively neglected. Maurissen's survey (*3*) of the overwhelming prevalence of agent effects on somatosensory function argues persuasively that the time is at hand to reverse this neglectful trend. For unknown agents, if they affect the nervous system at all, disruption of peripheral somatosensory function seems always to be a safe prediction.

Consequently, there is an immediate urgent need for the application of existing animal psychophysical methods to the somatosensory area and for innovative new approaches, particularly where time and cost advantages can be found.

Helpful discussions with P. M. Blough and, over a long period, with D. A. Eckerman and H. A. Tilson, are gratefully acknowledged. I thank Mrs. Nancy Mitchell for much assistance in the preparation of this work.

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Environmental Health Perspectives
Vol. 44, pp. 101-105, 1982

Environmental Factors Affecting Chemoreceptors: An Overview

by Bruce P. Halpern*

Vertebrate olfactory and gustatory receptors are necessarily exposed to the fluid which contains their relevant chemosensory environment. In terrestrial mammals, the nasal airways serve as protective accessory tissues for the olfactory receptors, but taste receptors in all vertebrates and olfactory receptors in fish are directly exposed to the liquids which bring chemosensory stimuli to them. The differentiated epithelial cells which form taste buds and the specialized neurons which are the vertebrate olfactory receptors are constantly replaced in normal adult animals, suggesting that chemosensory function per se is damaging to the receptors. Organic and sulfur-containing air pollutants may be among those which adversely affect olfactory receptors, but adequate data are not available. Surfactants and heavy metals can produce physiological and/or morphological damage in gustatory receptors. Some heavy metals are concentrated in saliva, a liquid which interacts closely with taste receptors. A failure to evaluate human chemosensory function in relation to potential chemosensory toxicants accounts for the present inability to specify the incidence or severity of the problem.

Introduction

Interaction with the chemical environment is the ultimate fulfillment for olfactory and taste receptors. As is often the case with such intimate and intense interaction, the results, while stimulating, can be damaging or destructive. Nonetheless, the continued presence and operation of these chemoreceptors indicate that mechanisms are available to cope with whatever morphological and/or physiological damage may be produced by common terrestrial and aquatic chemosensory environments. Additional environmental hazards derived from human culture (1-3) can, however, overwhelm the survival mechanisms of chemoreceptors (4, 5).

Olfaction

For terrestrial vertebrates, the environmental challenges to taste and olfactory receptors are rather different. Chemicals in solution or suspension in air comprise the major chemosensory hazards for olfaction in terrestrial forms. Since solids and liquids need not arrive at the receptor sheet, olfactory receptors can be so located that "accessory tissues" of the nose permit access only to gases. In mammals, in particular in humans, the sharp bends, narrow passages and extensive ciliated mucosal surface of the turbinates and septum of the nasal airways cause almost all particles larger than 5 μm to be deposited before they reach the olfactory receptors (6), which are located on the most superior turbinate and associated septum. During normal breathing, little of each inspiration flows above the middle turbinate, although a more even flow occurs during expiration. The large mucosal surface extending from the anterior nares absorbs over 90% of water-soluble vapors present in inspired air. Considerable adsorption of water-insoluble gases also occurs. Thus, the air flow pattern and mucosal sorption tend to protect the olfactory receptors against exposures to high concentrations of chemicals (6-9). Finally, the mucus-containing surface liquid and the rich blood supply of the nasal airways modulate the temperature and control the relative humidity of inspired air such that air temperature at the superior turbinate is within ±5°C of 37°C, and the relative humidity is 100%, for a wide range of ambient temperatures (*6*). The resulting "constant temperature and humidity chamber" for the olfactory receptors, which are also well protected from most nonvapor input, might seem to be a benign if not extraordinarily safe environment.

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If the olfactory receptors could interact with the chemosensory environment without incurring damage to themselves, we might expect these specialized neurons (*10, 11*) to be relatively static structures. However, many studies have found that the olfactory receptor cells are constantly being replaced (*10, 12, 13*), and that the entire receptor neuron can regenerate from basal cells in adult vertebrates, including birds and mammals. The regeneration produces not only morphological replacement but also functional recovery (*14*). These observations indicate that olfactory function is inherently damaging to the receptor. That is, the usual finding in healthy animals which have not been subjected to an intentionally polluted environment is constant replacement of the mucosal portion of the olfactory receptors. This receptor turnover, which must have a considerable metabolic cost, can provide an adaptive advantage only if it is necessary for satisfactory olfactory function.

Olfactory damage due to pollutants may result from an additional burden added to the challenge of normal olfaction. Structural and functional damage to the olfactory epithelium by ether or chloroform vapor was confirmed in the 1960s (*5*). Exposures to a sufficient concentration of either vapor for 6-10 min produced morphological damage, but the same concentrations for less than 4 min resulted in only a brief depression of function. However, ether and chloroform are not common environmental pollutants (*3*). Therefore, they may be useful research tools and possible model agents rather than problem chemosensory toxicants.

Sulfur compounds (*2*) and formaldehyde vapor (*1*) are common air pollutants. Olfactory deficits are reported to occur in humans exposed for some hours to formaldehyde vapor (*15*), but satisfactory psychophysical documentation is lacking. If formaldehyde vapor were an occupational hazard only for plastic, wood and textile workers, as well as anatomists, formaldehyde olfactory toxicity would be a potential problem for only a small segment of the population (*1*). However, the development and commercial use of formalin-based synthetic polymers for phenolic and urea resins have brought the challenge of this possible chemosensory toxicant to the general population (*1*). The problem may be more serious in urban settings, since formaldehyde occurs in gasoline and diesel engine exhaust, and as a photodecomposition product in smog. Irritant actions on the eyes and pulmonary airways are observed at air concentrations of formaldehyde no more than two to three times that needed to recognize by smell the presence of formaldehyde. Indeed, it is recommended that if a formaldehyde odor is consistently recognized in one's environment, a problem is likely to exist (*1*). Of course, if formaldehyde damages olfactory receptors, the recommended bioassay would be of limited value. Thus, an adequate evaluation of the severity of the effects of formaldehyde on olfaction is needed, with reliable dose-response profiles.

Irritant properties of sulfur dioxide are widely recognized (*16*). Although the presence of such irritants does increase airway mucus secretion (*17*), sulfur dioxide concentrations of 1 ppm or more impair nasal mucous flow (*6*). Surprisingly, continuous exposure to sulfuric acid aerosols with concentrations from 150 to 502 mg/m^3 for 7 days produced no nasal septum (or pulmonary) damage in rats or rhesus monkeys (*18*). Lesions were seen in guinea pigs; in mice, somewhat longer exposures resulted in ulcerations in the larynx and upper trachea. Since the highest concentrations in this study were several times that found in industrial pollutant-induced smog, generalization of sulfur-induced nasal damage from rodents to primates may be contraindicated. No evaluation of olfactory effects was made.

The central nervous system target of the olfactory receptor neuron axons, the olfactory bulb, apparently shows structural alterations in the rat as a function of the chemicals in inspired air (*19*). Initial reports suggested that bulb damage due to overstimulation was being observed. However, replication of the changes using the original stimulus paradigm has been difficult. Some reports suggest that the anatomical changes are signs of normal function, with unchanged olfactory bulb regions reflecting a pathological state due to insufficient input from the olfactory receptors (*19*). Finally, some investigators, while finding transient behavioral changes, have observed no alterations in olfactory bulb morphology (*20*).

In general, olfactory systems of terrestrial animals respond to, and permit adaptive behavior in relation to, very low concentrations of a broad range of molecules in air (*21*). This high and differential sensitivity uses "accessory tissues," the nonolfactory portions of the nasal airways, to humidify, temperature control and desaturate inspired air. The olfactory receptor neurons undergo constant turnover, and the entire neuron can develop in adult mammals from basal cells. These protective and replacement mechanisms may be insufficient to handle culturally produced air pollution. Formaldehyde vapor is potentially a serious olfactory receptor toxicant, but more data are needed. More broadly, any form of air pollution which adversely alters nasal mucosa, such as pH changes (*3*) or organic vapors (*1*), may also affect the nasal chemoreceptors.

The postsynaptic neurons of the olfactory bulb show histochemical changes following prolonged inspiration of air containing sufficient concentrations of a range of chemicals. Whether these changes, or their absence, are pathological is not understood.

Taste

Taste receptors of vertebrates occur in loci where contact with environmental chemicals in aqueous solution is a likely event (*22*). For fully aquatic forms such as fish, these loci include, in addition to the mouth, the exterior surface of the body, and sometimes appendages literally covered with taste buds (*4*, *23*). Other specialized "contact chemoreceptors," such as portions of the lateral line system and scattered chemoreceptors, are also found in fish and elasmobranchs. However, the receptor cells of these latter systems do not resemble those of taste buds, and the innervation may be either cranial or spinal nerves.

Since the fluid in which fish live is a liquid, olfaction and taste share the same stimulus solvent, and both can be affected by water pollution. For example, olfactory epithelium damage in fish due to exposure to petroleum in water has been reported (*24*).

Terrestrial vertebrates have taste receptors only within the mouth and pharynx. Adult humans and other mammals usually have taste buds on the tongue (generally on papillae located on the dorsal surface and sides of the tongue), the soft palate, and the epiglottis (*22*, *25*). Extensive protective and filtering accessory tissues comparable to the nasal airways are not found for vertebrate taste receptors. At best, a mucosal layer (*26*) [covering external taste buds in aquatic forms (*23*, *27*)] or a mixture of saliva and mucus (oral and pharyngeal taste buds) covers the receptor cell complex.

In humans and many other mammals, the tongue has a relatively thick and nonpermeable keratin layer (*28*). However, the keratin becomes very thin or disappears entirely in the vicinity of taste buds (*25*). An opening in the epithelium forms a pore (*28-31*) through which liquids, pastes and particles less than [illegible] µm can approach the microvilli which are the terminations of the receptor cells (*32*). These microvilli, and the distal ends of the taste bud receptor cells from which they project, are embedded in a matrix which may be a polysaccharide-protein mixture (*26*, *31*). The function of this pore chamber matrix is unknown, but it does not seem to present a substantial diffusion barrier (*28*, *33*). However, movement of chemicals from the pore chamber into either the taste buds cells or deeper into the taste buds does not readily occur (*28*), at least in living mammals (*31*). With prolonged contact, radiolabeled glycine, at least, can appear deep in the taste bud (*34*).

The relatively unprotected nature of taste receptors seems unavoidable if rapid but differential responses are to occur for a wide range of molecules and ions solvated in aqueous medium. One would expect that such exposure, which also can include a temperature range from 0°C to almost 40°C (potential terrestrial environment ambient range for drinking water sources) and small particle abrasion, would quickly damage the receptors. This seems to be the case, since the taste bud receptor cells of vertebrates undergo constant replacement (*28*). These cells continuously develop from epithelial cells, enter the taste bud, follow a sequence of differentiation, and disappear. In poikilotherms, the rate of cell turnover can be relatively slow (*12*) and is temperature-dependent. For mammals, a typical taste bud receptor cell has a life span of 10 days (*28*).

Water pollution damages taste receptors in fish. Surface active substances such as detergents appear to be one important factor (*4*, *35*); heavy metals, another (*36*). Mercurials reduce taste stimulus binding at relatively low concentrations, e.g., 100 μM $HgCl_2$. It may be that human taste receptors are also adversely affected by surfactants to which they are exposed. Situations such as detergents in water represent a pollution problem and are recognized as such. However, the surfactant sodium lauryl sulfate (dodecyl sodium sulfate), which is added to toothpaste (*37*), has been demonstrated to modify human taste perception (*38*), as does direct application of dentifrice to the tongue (*39*).

Saliva is a major component of the environment of mammalian taste receptors (*40*). Sufficient variations in its composition or quantity alter taste function. Thus, the ionic composition of saliva affects human taste judgments (*29*); a reduced supply modifies taste-dependent behavior (*40*), leads to altered neural responses and is associated with structural changes in taste bud cells (*32*).

A number of metals and other elements are concentrated in saliva (*41*). This phenomenon includes the heavy metals mercury and copper, and perhaps lead, as well as the halogen fluorine. It has given rise to the suggestion that saliva samples be used to monitor exposure to metal pollution in humans (*41*, *42*). Heavy metals have a variety of effects on living organisms (*16*). With reference to gustation, salivary heavy metal levels may be significant because taste receptors are sensitive to topical application of heavy metals (*34*). For example, 100 μM $CuCl_2$ depresses mammalian gustatory neural responses to sugars and amino acids. Since water supplies can be contaminated with heavy metals (*43*), human ingestion and subsequent salivary concentration is possible.

In general, the differentiated epithelial cells which make up vertebrate taste buds have little protection from the liquid-borne chemical and thermal events which are their stimuli. The constant and relatively rapid replacement of taste bud cells suggests a high likelihood of damage. Most gustatory stimuli probably do not penetrate farther than the taste bud's pore chamber. Surfactants are known to affect and sometimes damage taste buds, can occur as pollutants in water supplies, and are used as additives in oral hygiene products. Saliva has an important but not fully understood interaction with taste receptors. Since many heavy metals tend to concentrate in saliva, and taste responses can be altered or blocked by topical application of heavy metal solutions, exposure to heavy metal pollution may lead to gustatory damage.

Overview

Many potential environmental hazards exist for olfaction and taste. There has been a general failure to consider or evaluate chemosensory deficits as potential toxicological problems. The importance of human chemosensory function, and the many difficulties which result from chemosensory dysfunction, have been recognized (*44*), but the incidence of chemosensory toxicants in the environment is largely unknown. Adequate testing of chemosensory function as part of industrial medical screening, and as a component of more general examinations, is needed. Our present ignorance may be obscuring a sizeable array of preventable or correctable chemosensory disorders. An active and aggressive program is needed to clarify the nature of environmental factors affecting chemoreceptors.

I thank W. S. Cain and M. M. Mozell for discussions on the contents of this paper, and P. J. Canney and P. T. Halpern for critical comments on the manuscript. Research support was from NSF Grant BNS-8014148 during preparation of this report.

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Environmental Health Perspectives
Vol. 44, pp. 107-117, 1982

Gen tic Differences in Drug Metabolism Ass ciated with Ocular Toxicity*

by Hi oshi Shichi† and Daniel W. Nebert‡

The tissue localization and subcellular distribution of drug-metabolizing enzymes in the eye are described. With the use of inbred strains of mice, the [*Ah*] complex is shown to be an important experimental system for probing genetic differences in drug metabolism and related drug toxicities. Although the genetic system described in detail here involves mice, there is ample evidence that the same system operates in man.

Genetic differences in acetaminophen- and naphthalene-induced cataract formation and other ocular degeneration are shown to be related to the [*Ah*] complex. Because this toxicity appears similar to senile cataracts, we propose that certain types of drug-induced cataracts might exist among clinical populations of senile cataracts but that any cause-and-effect relationship would be very difficult to determine because of underlying interindividual differences in genetic predisposition. It is therefore suggested that genetic differences in drug metabolism be an important consideration in the clinical assessment of ocular toxicity caused by drugs and other environmental pollutants.

Introduction

Other contributions in this symposium are concerned with drug toxicity and pathology related to tissues of the head and neck. This paper deals more directly with the drug-metabolizing enzymes involved. These enzymes are responsible for toxification (i.e., potentiatio of the toxic response by forming a reactive dr g intermediate), as well as detoxication (metabolis leading to the excretion of innocuous products). This paper also emphasizes genetic difference in these enzymes, whereas genetics is not addres d in the other contributions. Although the geneti ystem described in detail here involves mice, ther s ample evidence that the same system operates i man. It therefore should be obvious that, due differences in genetic predisposition among in iduals in the human population, a particular se of an ophthalmic drug might be toxic to o person but not to another (and these two persons might even be siblings). Similar genetic differences in toxicity caused by drugs and other environmental pollutants are expected to be manifest among other tissues of the body, as well as in the eye.

We first describe the tissue localization and subcellular distribution of drug-metabolizing enzymes in the eye. Next, the genetic system, called the [*Ah*] complex, is introduced. Finally, genetic differences in acetaminophen- and naphthalene-induced cataract formation (lens opacification) and other ocular degeneration are shown to be related to the [*Ah*] complex.

Concept of Extrahepatic Drug Metabolism

Until the last two decades, it was generally accepted that the liver was the only important organ for drug metabolism. More recently, however, it has become clear that virtually every cell of every plant and animal (and certain bacteria) contains drug-metabolizing capability. At least part of this appreciation reflects the development of much more sensitive assays for drug-metabolizing enzymes.

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*This pap vas not presented at the Symposium on Target Organ Toxic Eye, Ear and Other Special Senses.

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Toxification and detoxication of drugs and other environmental pollutants in extrahepatic tissues therefore can be important (*1*).

The eye is no exception. For example, chloroquine retinopathy observed in malaria patients was attributed to an accumulation of unusually high levels of chloroquine in the eye (*2*). Unless the eye possesses a mechanism for removing such drugs that enter ocular tissues via the circulating blood, therefore, accumulated chemicals of all types might exert adverse effects on photoreceptor cells, leading to visual impairment. Shortly after Bernstein's report (*2*), cytochrome P-450, a principal component of the drug-metabolizing enzyme system,* was detected spectrally in the pigmented epithelium of bovine retina (*4*). Although much progress has been made in studies on absorption, distribution, and clinical usefulness of ophthalmic drugs (*5*), information on ocular drug metabolism and toxicity remains quite scanty, in spite of obvious clinical importance.

Futher, information about genetic differences in ocular drug toxicity is even more scarce. Steroid-induced glaucoma is a pharmacogenetic disorder, for example, although the etiology is unknown. About 5% of the United States population is homozygous for the recessive allele causing glaucoma induced by corticosteroid ophthalmic medications (*6*).

P-450-Mediated Monooxygenase Activities and Other Coordinated Drug-Metabolizing Enzymes

Many environmental pollutants and other foreign compounds are chemicals that are so hydrophobic they would remain in the body indefinitely were it not for the metabolism resulting in more polar derivatives. These drug-metabolizing enzyme systems, which are localized principally in the liver, are usually divided into two groups: phase I and phase II. During phase I metabolism, one or more polar groups (such as hydroxyl) are introduced into the hydrophobic parent molecule, thus allowing a handle, or position, for the phase II conjugating enzymes (such as UDP glucuronosyltransferase) to attack. The conjugated products are sufficiently polar, so that these detoxified chemicals are now excreted from the body (*7*).

One of the most interesting of the phase I enzyme systems is a group of enzymes known collectively as the P-450-mediated monooxygenases ([illegible]). These membrane-bound enzyme systems metabolize polycyclic aromatic hydrocarbons such as benzo[a]pyrene (ubiquitous in city smog, cigarette smoke and characoal-cooked foods) and biphenyl; halogenated hydrocarbons such as polychlorinated and polybrominated biphenyls, insecticides, and ingredients in soaps and deodorants; strong mutagens such as *N*-methyl-*N*′-nitro-*N*-acetylarylamines and nitrofurans; numerous aromatic amines, such as those found in hair dyes; nitro aromatics and heterocyclics; wood terpenes; epoxides; carbamates; alkyl halides; safrole derivatives; certain fungal toxins and antibiotics; many of the chemotherapeutic agents used to treat human cancer; most drugs; small chemicals such as benzene, thiocyanate, or ethanol; both endogenous and synthetic steroids; and other endogenous compounds such as biogenic amines, indoles, thyroxine, and fatty acids.

Evidence is growing that metabolism to reactive intermediates by cytochrome P-450-mediated monooxygenases is a prerequisite for mutagenesis, carcinogenesis, and toxicity caused by numerous drugs, polycyclic hydrocarbons, and other environmental pollutants. The steady-state levels of these reactive electrophilic intermediates and, consequently, the rates at which they interact with the critical nucleophilic target, are dependent upon a delicate balance between their generation and detoxication (Fig. 1). Changes in the balance between toxification and detoxication in any particular tissue of an individual may therefore affect his risk of tumorigenesis or toxicity.

Subcellular Localization and Distribution of Drug-Metabolizing Enzymes in the Eye

The distribution (Table 1) and subcellular localization (Table 2) of several drug-metabolizing enzyme activities were studied in bovine eye. AHH activity† was chosen as an example of phase I metabolism (reflecting basal forms of P-450). UDP glucuronosyltransferase, glutathione transferase, and *N*-acetyltransferase activities are typical phase II enzymes (*9*). γ-Glutamyltranspeptidase and cystine

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*In this paper, cytochrome P-450 is defined as all forms of CO-binding hemoproteins associated with membrane-bound NADPH-dependent monooxygenase activities. We define cytochrome P_1-450 as all forms of CO-binding hemoprotein that increase in amount concomitantly with rises in induced AHH activity following polycyclic aromatic inducer treatment. In view of more than one such form of P_1-450 (*3*), it is emphasized that this definition of P_1-450 is simplistic.

†Abbreviations used include: AHH, aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC 1.14.14.1); B6, the C57BL/6N inbred mouse strain; D2, the DBA/2N inbred mouse strain.

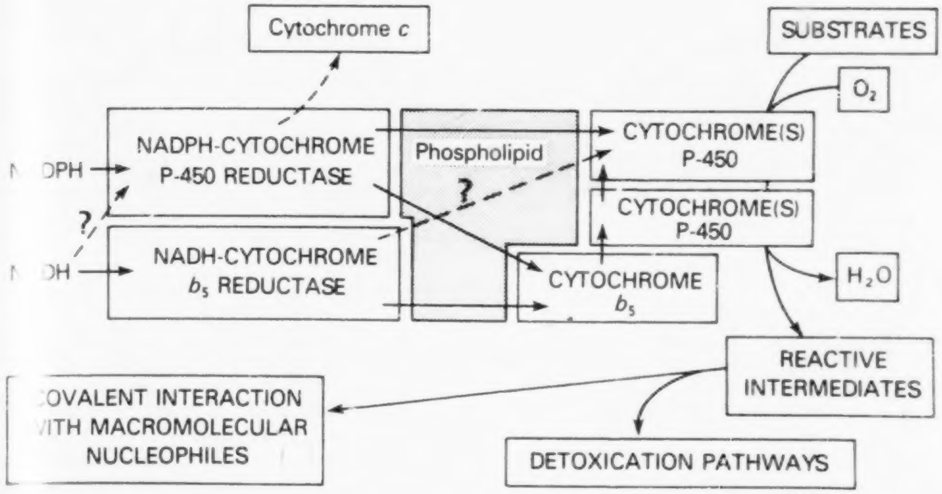
Cytochrome *c*
SUBSTRATES
O_2
NADPH
NADPH-CYTOCHROME P-450 REDUCTASE
Phospholipid
?
CYTOCHROME(S) P-450
CYTOCHROME(S) P-450
?
NADH
NADH-CYTOCHROME b_5 REDUCTASE
CYTOCHROME b_5
H_2O
REACTIVE INTERMEDIATES
COVALENT INTERACTION WITH MACROMOLECULAR NUCLEOPHILES
DETOXICATION PATHWAYS

FIGURE 1. Scheme for the membrane-bound multicomponent monooxygenase system(s) and the various possibly important pathways for hydrophobic substrates (*8*). For any given substrate, the relative balance between metabolic activation and detoxication likely would differ among different tissues, strains, and species. Age, genetic expression, nutrition, hormone concentration, diurnal rhythm, pH, saturating versus nonsaturating conditions of the substrate, K_m and V_{max} for each enzyme, subcellular compartmentalization of each enzyme, efficiency of DNA repair, and the immunological competence of the animal—may all be important factors affecting this balance.

Table 1. Distribution of AHH, UDP glucuronosyltransferase and γ-glutamyltranspeptidase activities in various tissues of bovine eye.

Tissues	Enzyme activity, units/mg protein[a]		
	AHH	UDP Glucuronosyltransferase	γ-Glutamyltranspeptidase
Retina	0.03	6	400
Lens	Not detectable	5	3
Cornea	0.02	8	320
Iris	0.15	23	640
Pigmented epithelium-choroid	0.31	84	430
Ciliary body	5.74	396	3850

[a] Tissue homogenates were used for assay of enzymic activities. One unit of activity is the amount of enzyme protein that produces 1 pmole of product per minute. Enzyme activities given here are specific activities, i.e., units/mg of tissue protein/minute.

Table 2. Subcellular activities of enzymes involved in mercapturate synthesis in the bovine eye.[a]

Subcellular fraction	Glutathione transferase[b]		γ-Glutamyltranspeptidase		Cystine aminopeptidase		*N*-Acetyltransferase	
	Ciliary body	Pigmented epithelium-choroid	Ciliary body	Pigmented epithelium-choroid	Ciliary body	Pigmented epithelium-choroid	Ciliary body	Pigmented epithelium-choroid
Tissue homogenate	5210	480	3850	430	100	8	13	2
Nuclei	780	230	270	67	1	Not detectable	7	1
Mitochondria	1400	120	1620	310	73	7	64	2
Microsomes	3690	590	5120	570	1130	54	160	8
Supernatant	6340	870	1230	130	450	13	13	Not detectable

[a] Activities are expressed as units/mg protein. See Table 1 for definition of one unit of activity.
[b] With 1-chloro-2,4-dinitrobenzene as the substrate.

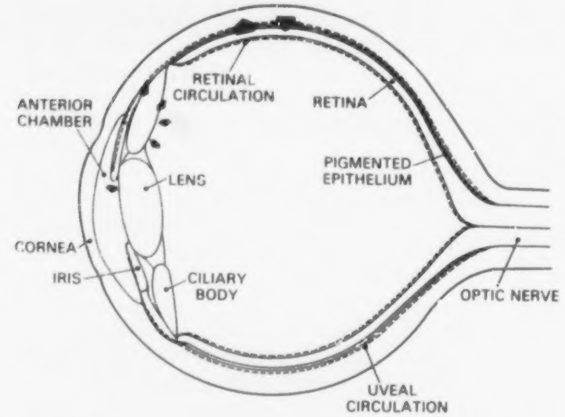

FIGURE 2. Retinal and uveal blood circulation systems (dashed lines). The retinal circulation provides nutrients for the retinal neurons, while the uveal circulation supplies nutrients for the pigmented epithelium (and visual cells), the ciliary body (and lens and cornea), and the iris. Large arrows denote entry of nutrients and exit of metabolic products between the uveal circulation (choroidal capillaries) and the pigmented epithelium. Small arrows indicate aqueous humor secretion by the ciliary body into the anterior chamber and aqueous humor.

aminopeptidase are important in the pathway from glutathione conjugates of aromatic hydrocarbons to mercapturate formation (*10*). Most, if not all, of these six enzymes (Tables 1 and 2) are induced by various drugs or other environmental pollutants (*3*, *9*, *10*). Low levels of γ-glutamyltranspeptidase (*11*) and glutathione transferase (*12*) have been reported in the lens.

We conclude from Tables 1 and 2 that virtually all ocular tissues have detectable drug-metabolizing capability (*13-15*). The ciliary body and pigmented epithelium-choroid are by far the richest in these activities (see Fig. 2 for the location of these tissues in the eye). In fact, the specific activities of AHH and UDP glucuronosyltransferase in the ciliary body are only about 10 to 20 times less than those in bovine liver.

The [*Ah*] Complex: Genetic Expression of Induced AHH Activity and P_1-450 Induction

The [*Ah*] complex is an experimental model system that has provided several good examples of a delicate balance between genetic and environmental factors in the etiology of cancer, drug toxicity and birth defects (*16*). The [*Ah*] complex of

Table 3. Induction of ocular AHH activity in B6 d D2 mice by polycyclic aromatic compounds.

	Specific AHH tivity, units/mg p ein[a]	
Intraperitoneal treatment	B6	D2
Control	0.08	0.06
β-Naphthoflavone	0.36	0.07
3-Methylcholanthrene	0.39	0.08
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	0.47	0.10

[a] See Table 1. Tissue homogenates of the enti eye were prepared for the enzyme assay.

the mouse regulates the induction (by olycyclic aromatic compounds such as 3-methylchol nthrene, benzo[a]pyrene, or 2, 3, 7, 8-tetrachloro benzo-*p*-dioxin) of numerous drug-metabolizing enzy ne "activities" associated with several (or many) ne induced forms of cytochrome P_1-450. The [*Ah*] complex comprises numerous regulatory, structural, and probably temporal genes that may or may not be on the same chromosome.

At any given dose of inducer, the induction of AHH activity and at least two dozen other monooxygenase activities and associated forms of P_1-450 occurs in 3-methylcholanthrene-treated B6 and other genetically "responsive" inbred strains and is always much lower in 3-methylcholanthrene-treated D2 and other genetically "nonresponsive" strains. Besides the liver, this genetic expression is seen in such tissues as lung, kidney, intestine, lymph nodes, skin, bone marrow, pigmented epithelium of the retina (Table 3), brain, mammary gland, uterus, ovary and testis. The genetic response is herefore called "systemic," or occurring throughout virtually all tissues of the animal. Responsivene to aromatic hydrocarbons has been designated he [*Ah*] complex: Ah^b is the dominant allele; A is the recessive allele; the Ah^b/Ah^d heterozygo is phenotypically similar to the Ah^b/Ah^b mouse n terms of degree of responsiveness. The trai of *Ah*-responsiveness among crosses involving and D2 mice is therefore autosomal dominant (*16*

Several studies indicate that the fur amental genetic difference resides in the *Ah* r ulatory gene (Fig. 3), which encodes the cytosolic eceptor capable of binding to inducers such as methylcholanthrene, benzo[a]yprene, and 2,3, 8-tetrachlorodibenzo-*p*-dioxin. To our knowledge, nly certain foreign chemicals bind to this rece or with saturability and high affinity (less than nmole). The B6 receptor appears to have at least 0 times better affinity toward inducers of P_1-450 han the Ah^d/Ah^d mouse (*18*). Translocation of the nducer-

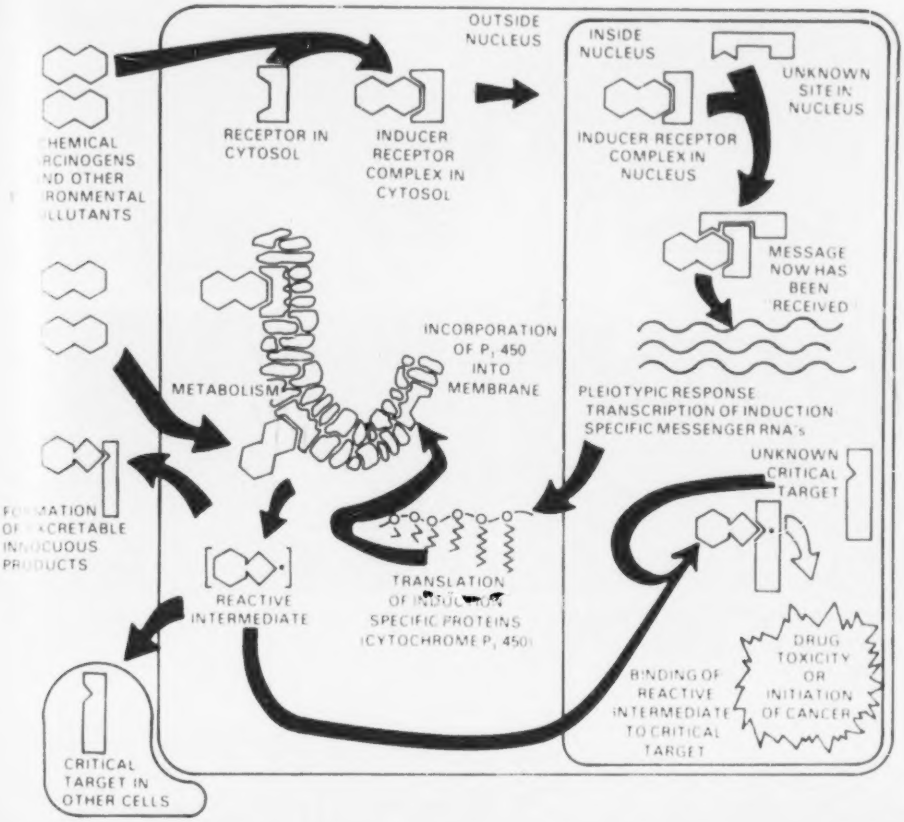
OUTSIDE NUCLEUS
INSIDE NUCLEUS
HEMICAL
RCINOGENS
ND OTHER
RONMENTAL
LLUTANTS
RECEPTOR IN CYTOSOL
INDUCER RECEPTOR COMPLEX IN CYTOSOL
UNKNOWN SITE IN NUCLEUS
INDUCER RECEPTOR COMPLEX IN NUCLEUS
MESSAGE NOW HAS BEEN RECEIVED
INCORPORATION OF P_1 450 INTO MEMBRANE
METABOLISM
PLEIOTYPIC RESPONSE TRANSCRIPTION OF INDUCTION SPECIFIC MESSENGER RNA's
UNKNOWN CRITICAL TARGET
FO MATION OF XCRETABLE IN CUOUS PR UCTS
REACTIVE INTERMEDIATE
TRANSLATION OF INDUCTION SPECIFIC PROTEINS (CYTOCHROME P_1 450)
DRUG TOXICITY OR INITIATION OF CANCER
BINDING OF REACTIVE INTERMEDIATE TO CRITICAL TARGET
CRITICAL TARGET IN OTHER CELLS

FIGURE 3. Diagram of a cell and the hypothetical scheme by which a cytosolic receptor, product of the regulatory *Ah* gene, binds to inducer (*17*). Depending upon the half-life of the reactive intermediate, the rate of formation of the intermediate, and the rate of conjugation and other means to detoxify the intermediate—important covalent binding may occur in the same cell in which metabolism took place, or in some distant cell. Although the "unknown critical target" is illustrated here in the nucleus, there is presently no experimental evidence demonstrating unequivocally the subcellular location of a "critical target(s)" required for the initiation of drug toxicity or cancer. (Reproduced with permission).

receptor c plex into the nucleus has been demonstrated in he *Ah*-responsive heterozygote and homozygo (*18*) and requires a temperature-dependent tep (*19*). Discrepancies between the dextran-ch coal adsorption assay and the sucrose density g ient assay have been recently understood via omatographic studies of the *Ah* receptor (*20*).

What h pens in the nucleus is not yet known (Fig. 3), b somehow the "information" (that these inducers P_1-450 exist in the cell's microenvironment) is eived; the response is transcription of specific m A's, translation of these mRNA's into specific e mes such as P_1-450, and incorporation of P_1-450 to cellular membranes. These induced enzymes ay aid in detoxication or they may generate increased amounts of reactive intermediates.

The *Ah* Complex in Human Populations

In spite of shortcomings with the AHH assay in human cultured lymphoblasts (*8*, *21*), a growing list of clinical disorders appears to be associated with the human [*Ah*] complex. There clearly exists sufficient evidence that heritable variation of AHH inducibility occurs in man. Experimental difficulties, however, make it impossible at this time to be certain whether AHH induction is controlled by one or more genetic loci.

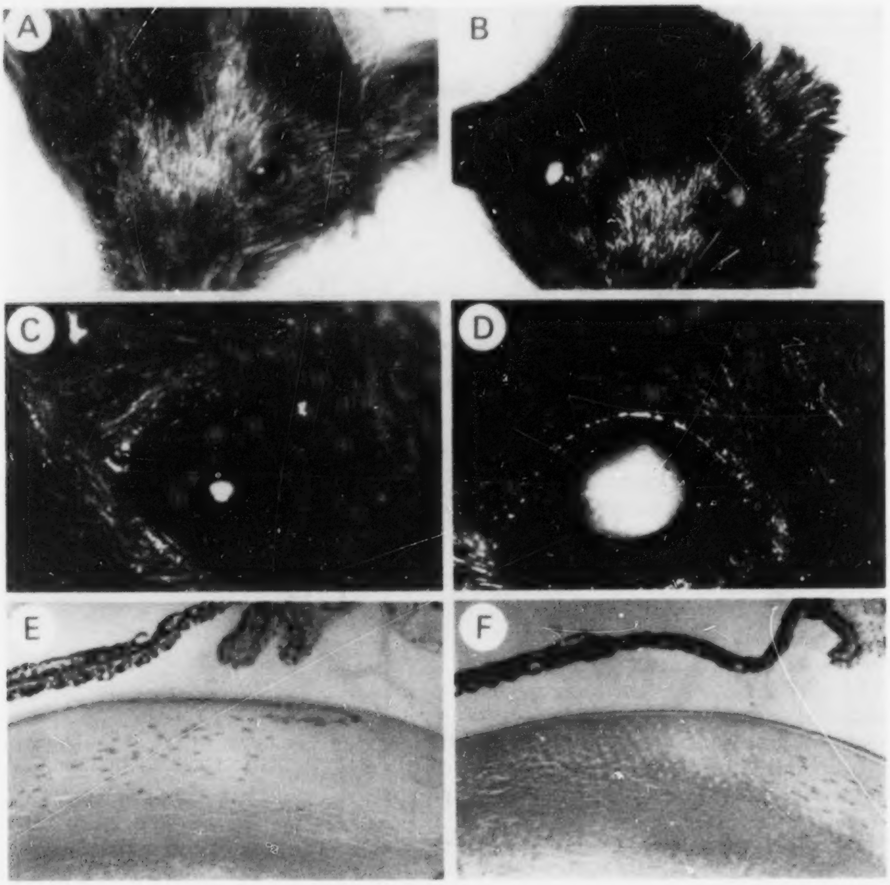

FIGURE 4. (*A*, *C*) Genetically nonresponsive Ah^d/Ah^d homozygote, and (*B*, *D*) genetically responsive Ah^b/Ah^d heterozygous sibling from the $B6D2F_1$ × D2 backcross (*23*). These photographs were taken 5 hr after the mice had received acetaminophen and 53 hr after the mice had received 3-methylcholanthrene, an inducer of P_1-450. (*E*, *F*) Hematoxylin- and eosin-stained sections of ocular tissue from mice in (*A*) and (*B*), respectively (× 500). The tissues from top to bottom are iris, cornea and lens. Vacuoles seen in the subepithelial layer of the lens in (*F*) are the result of hydration of the lens cells and are always associated with cataract formation (*24*). Doses of more than 1000 mg of acetaminophen/kg body weight were almost always fatal to Ah^b/Ah^d mice within the first 8 hr after acetaminophen administration, but these doses were not lethal, nor did they ever cause lens opacification to Ah^d/Ah^d mice. At lower doses of acetaminophen (400 to 800 mg/kg), the ocular opacity developed more slowly in Ah^b/Ah^d mice. If a cataract did not appear within 10 hr after acetaminophen administration, however, no cataract developed subsequently. The degree of opacification that had developed within these 10 hr was never reversible. (Reproduced with permission).

Correlation between the Ah^b Allele and Lens Opacification Caused by Intraperitoneal Acetaminophen

Acetaminophen, a widely used analgesic-antipyretic agent, is metabolized to a toxic intermediate largely by some form of polycyclic aromatic-induced P_1-450 (*16*). Therefore, we theorized that *Ah*-responsive mice—following treatment with polycyclic aromatic inducers to enhance P_1-450 levels—would display more acetaminophen toxicity than *Ah*-nonresponsive mice. This hypothesis turns out to be true not only in liver, where hepatic necrosis and covalent binding of acetaminophen metabolites occur (*22*), but also in the lens, where cataract formation was found (Fig. 4). An absolute correlation is found (Fig. 5) between lens opacification and the Ah^b allele among four *Ah*-responsive inbred mouse strains, five *Ah*-

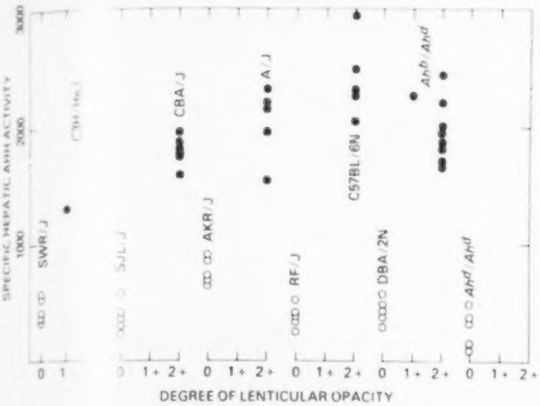

FIGURE 5. Correlation between the hepatic AHH inducibility and cataractogenesis among five nonresponsive inbred strains (○), four responsive inbred strains (●), and the (●) responsive (Ah^b/Ah^d) and (○) nonresponsive (Ah^d/Ah^d) progeny from the B6D2F$_1$ × D2 backcross (*25*). Each symbol represents an individual mouse. All mice had received 3-methylcholanthrene intraperitoneally 48 hr prior to intraperitoneal acetaminophen. The eyes were evaluated with an ophthalmic slit lamp 5 hr following acetaminophen treatment: ○ = no signs of opacification; 1+ = about 50% opacification; 2+ = complete opacification. The mice were then immediately killed, and the liver microsomal hydroxylase activity was determined.

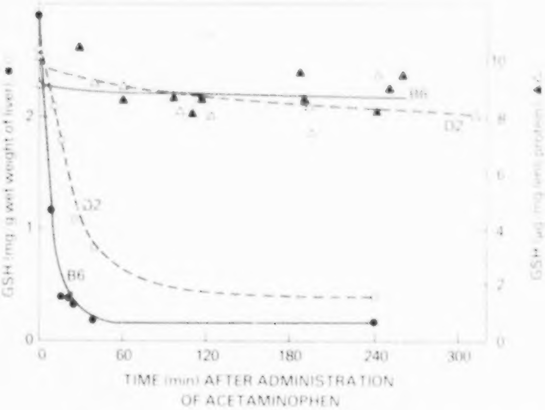

FIGURE 6. Total glutathione (GSH) levels in (○, ●) the liver and (△, ▲) lens of 3-methylcholanthrene-treated B6 mice (●, ▲) and D2 mice (○, △) following a large dose (1,000 mg/kg) of intraperitoneal acetaminophen (*25*).

nonresponsive inbred strains and among children of the B6D2F$_1$ × D2 backcross.

Figure 6 illustrates the effects of acetaminophen on glutathione concentrations in the liver and lens of 3-methylcholanthrene-pretreated B6 and D2 mice. As expected, hepatic glutathione depletion is more pronounced in B6 than in D2 mice. Quite unexpectedly, however, lenticular glutathione levels are not depleted in either B6 or D2 mice following the large intraperitoneal dose of acetaminophen.

One possibility is that acetaminophen and its metabolites do not reach the lens and therefore glutathione is not depleted. This possibility was ruled out with the use of radiolabeled acetaminophen (Fig. 7). Similar kinetics of covalent binding occurs in both liver and lens protein, and in each case B6 tissues exhibit two to five times greater covalent binding than the corresponding D2 tissues.

How can covalent binding of acetaminophen metabolites occur in the apparent absence of any glutathione depletion? At least two possible answers come to mind. First, assuming glutathione depletion in the lens is necessary for cataract formation, one might envision some sort of compartmentalization of glutathione. In other words, although total lens glutathione concentrations are similar in B6 and D2 mice, a subcellular depletion of glutathione (directly in the anterior subcapsular region of the lens where the cataracts develop) might occur in B6 but not in D2. Such a localized depletion might be augmented by a sudden influx of reactive intermediates (generated by the liver, ciliary body or whatever) so that local concentrations of the toxic agent far exceed local concentrations of glutathione in and near the anterior chamber. Alternatively, ocular toxicity may develop by a mechanism independent of glutathione levels. Electrophilic reactive intermediates could cause changes in cellular macromolecules in many ways: alterations in oxidations and reductions of endogenous molecules, free radical formation, permeability changes in the lens membrane, alterations in metabolism of endogenous substrates, etc.

Very large doses of acetaminophen were used for the experiments illustrated in Figures 4-7. The only clinical situation that such large doses might be achieved would be attempted suicide. Relevant observations, however, have been recently reported. Upon hearing a seminar about the data in this report, Cohen and Burk (*26*) examined ten patients showing evidence of acetaminophen-induced hepatotoxicity due to self-inflicted overdoses. The age range was 17 to 45 years old, with only two women more than 23 years of age. One of these patients, a 45-year-old alcoholic about whom no previous medical history was available and who died 1 day later, was found on admission to have bilateral diffuse cataracts; no previous history of cataracts was known. She also was the only fatality in the series of 10 cases (*26*).

Table 4. Effect of oral acetaminophen or naphthalene on cataract formation in B6 and D2 mice.

Inbred strain	Intraperitoneal P1-450 inducer treatment[a]	Oral treatment[b]	Drug concentration, mg/ml[c]	Mean body weight[d]			Incidence cataracts, %	Comments
				Day 0	Day 21	Day 60		
B6	3-Methyl-cholanthrene	Acetamino-phen	5	15.7 (15)	17.1 (13)	(0)	13.3	Two developed bilater about day 10 and b 1 to 2 weeks therea
D2	″	″	5	14.6 (15)	16.9 (15)	(0)	0	No cataracts at time
B6	3-Methyl-cholanthrene	Acetamino-phen	10	16.2 (15)	17.6 (12)	(0)	13.3	Two developed bilater about day 3 and bo within the next 2 w thereafter
D2	″	″	10	15.9 (15)	17.7 (15)	(0)	0	No cataracts at time
B6	β-Naphtho-flavone	Acetamino-phen	5	16.4 (15)	19.9 (15)	21.5 (15)	6.7	One developed a unil cataract about day examined when mo killed 7 months lat
D2	″	″	5	15.4 (15)	20.5 (15)	22.8 (14)	0	No cataracts
B6	β-Naphtho-flavone	Acetamino-phen	10	15.9 (15)	20.8 (14)	22.3 (4)	13.3	One developed a unila cataract about day 11 developed bilateral about day 14; both day 36
D2	″	″	10	16.9 (15)	21.6 (14)	23.9 (14)	0	No cataracts
B6	β-Naphtho-flavone	Naphthalene	5	15.4 (15)	19.4 (14)	20.7 (13)	6.7	One developed bilateral about day 27; these examined when mous killed 6 months later
D2	″	″	5	16.5 (15)	20.3 (15)	21.3 (15)	0	No cataracts
B6	β-Naphtho-flavone	Naphthalene	10	15.4 (15)	17.2 (13)	18.8 (12)	6.7	One developed a unilat cataract about day 8; examined when mous killed 7 months later
D2	″	″	10	16.5 (15)	16.7 (15)	19.4 (15)	0	No cataracts

[a] Injections twice weekly (*25*).

[b] The drug was dissolved in corn oil and the food was soaked in this solution of corn oil.

[c] Estimated drug concentration in corn oil in which the food was soaked.

[d] Mean body weights of group during feeding of the drug. The number of survivors is shown in parentheses.

Effect of Daily Oral Acetaminophen or Naphthalene on Cataract Formation

In additional experiments, we chose oral doses of acetaminophen, lower doses that are used clinically (Table 4). Oral naphthalene was also chosen for study because its monooxygenation is also catalyzed by some form of P_1-450 and associated with the *Ah* allele (*16*). Naphthalene-induced cataracts have been described (*27*) in workers from dye and chemical factories. The cataractogenic agent is claimed (*28*) not to be naphthalene itself but rather 1,2-naphthoquinone, which is formed from naphthalene-1,2-dihydrodiol. This diol of naphthalene reaches the eye via the bloodstream. Pathways of
naphthalene metabolism have been extensiv
tigated both *in vivo* (*29*) and with liver mi
in vivo (*30-32*). The phenol and the react
oxide of naphthalene and acetaminophen a
gated with glucuronide, sulfate, and glut

Relatively small numbers of B6 mice
detectable cataracts when acetaminophen
thalene is ingested daily (Table 4). Phen
induces these conjugating pathways, and
may explain our finding (*25*) that oral phen
can completely prevent acetaminophen-inc
aract formation. It is possible that the
intermediate of acetaminophen is an arene
a quinone. No D2 mouse ever develope
minophen- or naphthalene-induced catar
mice treated with 3-methylcholanthrene di
6 weeks, apparently due to the cytotoxici

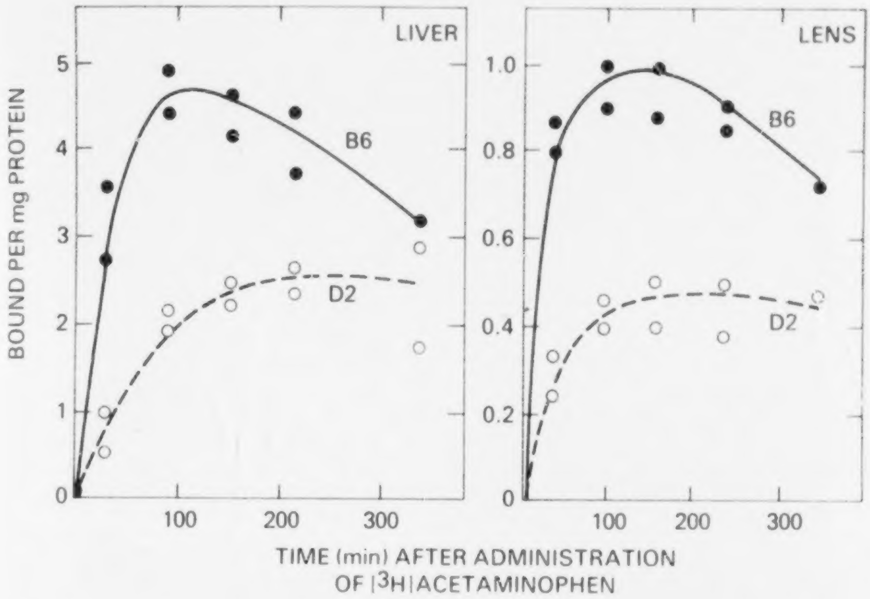

FIGURE 7. Covalent binding of [^{3}H]acetaminophen metabolite(s) to liver and lens proteins of 3-methylcholanthrene-treated (●) B6 and (○) D2 mice (*25*).

chemical when injected twice weekly. On the other hand, β-naphthoflavone treatment was not very toxic; relatively few animals died during 60 days of daily oral acetaminophen or naphthalene combined with intraperitoneal β-naphtoflavone twice weekly. Out of the nine B6 mice that developed cataracts on these various oral regimens, three remained otherwise healthy for 6 or 7 months, at which time the animals were killed in order to examine histologically the lenticular opacities.

Histology of Ocular Toxicity Induced by Oral Acetaminophen

Upon close microscopic examination, we found that tissue degeneration occurs not only in the anterior lens cortex but also in large areas of the uveal tract (Fig. *8a, c*). The retina and pigmented epithelium are normal (Fig. *8b*). The degeneration seen in the choroid, ciliary body and iris appears to be of a chronic nature. Anterior synechiae and inflammatory cells at the angle are frequently observed. The number of epithelial cells is usually decreased in cataracts that were young (several hours or several days old). Intermittent loss of cells from the lens epithelial layer is seen in cataracts that had been present for at least 1 week (Fig. *8c*). The gradual loss of the epithelial cells might result in metabolic derangements in the lens fibers, thereby leading to lens opacification. All lenses from D2 mice similarly treated do not show any evidence of ocular abnormalities.

Possible Interrelationship among Genetic Differences in Drug Metabolism, Drug-Induced Cataracts and Senile Cataracts in the Human Population

Cataracts induced by chemicals and drugs, especially naphthalene, have been extensively investigated because of their similarity to senile cataracts (*28, 33, 34*). The problem of trying to assess ocular toxicity in elderly patients receiving large daily doses of acetaminophen would be far more difficult than to study patients attempting suicide with acetaminophen, because of the combination of genetic factors (i.e., the [*Ah*] complex in man) and environmental factors (i.e., history of cigarette smoking, other drugs prescribed, dietary intake, etc.). Perhaps there exist other drugs in this same category: they may cause ocular toxicity in a

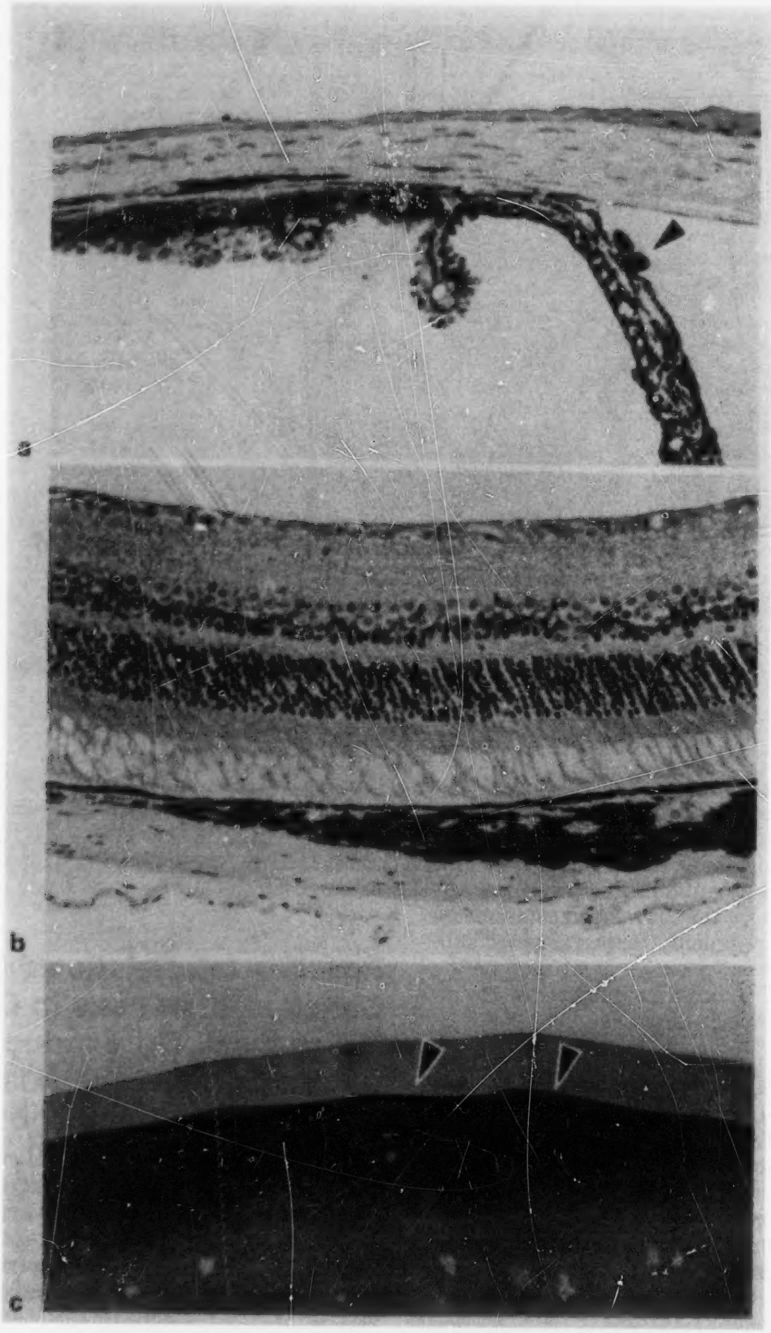

FIGURE 8. Hematoxylin- and eosin-stained sections of ocular tissues from B6 mice that had received acetaminophen daily in the diet for more than 1 month (25). (*a*) Light micrograph showing ciliary body, iris and trabecular meshwork. Note the presence of inflammatory cells in the angle (arrowhead) and the degenerative changes in the ciliary body. An atrophic iris and a certain amount of cellular infiltration of the episclera are observed. A peripheral anterior synechia is also seen. × 200. (*b*) Light micrograph showing the retina and choroid. Chronic degeneration of the choroid was frequently seen. The retina shows an almost normal appearance. × 200. (*c*) Light micrograph of the anterior cortical zone of the cataractous lens. Note the intermittent loss of epithelial cells from the epithelial cell layer (arrowheads). × 230.

geneticall determined small proportion of the total human p ulation. We do suggest, however, that the ocula xicity data shown in this report may be clinically portant to certain patients receiving either a s gle large overdose of acetaminophen or high dos ver a long period of time.

The exp ecretarial assistance of Ms. Ingrid E. Jordan is greatly app ated.

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Environmental Health Perspectives
Vol. 44, pp. 119-127, 1982

Review of Environmental Factors Affecting Hearing

by John H. Mills* and Jacquelyn A. Going*

The major nongenetic causes of sensorineural hearing loss are exposure to noise, aging, ototoxic drugs, viral and bacterial infections, and interactions between these factors. Regarding exposure to continuous noise, the data base from laboratory and field studies indicates that a risk of hearing loss is present when noise levels exceed 75–80 dBA. As noise level, duration and number of exposures increase so does risk. The data base for other forms of noise (intermittent, impact) is not as established. Risk of hearing loss due to impulse noise increases as the peak SPL exceeds 145-155 dB and as the duration of the impulse, the number of impulses and the number of exposures increase. High-level acoustic impulses can cause severe, permanent hearing loss. Interaction between some steady-state noises and some acoustic impulses can be synergistic, producing extensive injuries to the organ of Corti. Noise can also interact synergistically with some aminoglycoside antibiotics to produce severe injuries to the inner ear. These antibiotics are also capable of producing hearing loss and indeed may do so in up to 55% of the one million persons who receive aminoglyocisid antibiotics during the course of treatment for tuberculosis or severe gram-negative infections. Bacterial and viral infections may also produce mild to severe hearing loss. With the development of rubella vaccine and Rhogam, cytomegalovirus may have become the most common cause of congenital deafness. Aging is also a major cause of hearing loss. Exposure to occupational and environmental noise, certain diseases and life styles (diet, stress, drugs) may interact with the specific effects of aging. The result is moderate to severe hearing loss in a majority of older persons.

Introduction

This paper reviews major environmental factors which can affect hearing. Its focus is on those factors which produce injury to the cochlea and auditory nerve. Hearing losses arising from pathology of the cochlea and auditory nerve are referred to as sensorineural as opposed to conductive (external ear and middle ear) or central (CNS). The major causes of sensorineural hearing loss are exposure to noise, aging, drugs, genetic factors and a number of other factors including infections, head trauma, metabolic disorders and chemicals. Here, the major emphasis is on the effects of noise on hearing with minor attention given to aging, drugs, some of the other agents and some of the major interactions which can occur.

Noise and Hearing

Exposure to noise can injure the inner ear and produce a sensorineural hearing loss. The injury and hearing loss can be temporary, permanent, or it can have temporary and permanent components. Since World War II considerable effort has been given to studies of noise-induced hearing loss. (*1*) These efforts include large-scale field studies of permanent effects in occupational settings (*2-5*), laboratory investigations of temporary effects with humans (*6-8*), and laboratory investigations with animals of temporary and permanent effects (*9-11*), including pathological anatomy and physiology (*12*, *13*). Often the effects of noise on hearing are discussed in relation to the characteristics of the noise exposure. Three characteristics are discussed here: (1) continuous exposure to noise, (2) intermittent exposure and (3) impulse noise.

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Continuous Exposure to Steady-State Noise

Laboratory Studies. Recent studies in human subjects (*7, 14-17*) of temporary changes in hearing (called temporary threshold shifts) produced by exposure to noise are consistent in several respects. Temporary threshold shifts (TTS) increase during the first 8-12 hr of a noise exposure and then reach an asymptote or plateau (Fig. 1). TTS's at asymptote increase about 1.7 dB per decibel increase in noise level above a critical level (*7*) as shown in Figure 2. The critical level of a noise centered at 4.0 kHz is 74 dB sound pressure level (SPL). According to the simple equation in Figure 2, then, the TTS is 0 dB when the critical level *C* is equal to the noise level. For other noises between 500 Hz and 4.0 kHz, TTS is computed by the same equation. That is, TTS increases about 1.7 dB per decibel increase in noise level above the critical level, which is dependent upon frequency (Fig. 3). The critical level is 74 dB at 4.0 kHz, 78 dB at 2.0 kHz, and 82 dB SPL at 1.0 and 0.5 kHz.

Also shown in Figure 3 are data from laboratory investigations with chinchillas and monkeys (7). Figure 3 shows that the relationship between temporary hearing loss and noise level described for humans is also accurate for chinchilla and monkey data when noise level is corrected by a constant (the critical level). Critical levels are frequency and species dependent. It appears, therefore, that the equations in Figure 3 are accurate for humans, chinchillas, some species of monkey and perhaps most mammals.

Several years ago it was hypothesized (*14*) that TTS at asymptote produced by a given sound is the upper limit of any permanent effect that can be produced by that sound regardless of the duration of scheduling of the exposure. This hypothesis is correct if TTS grows to an asymptote rather than an intermediate plateau. Research with chinchillas (*20, 21*) and monkeys (*11*) shows that the asymptote is maintained for as long as 90 (*11, 20*) or 108 days (*21*). Thus, if a noise produces a barely measurable TTS (5 dB) after about 12 hr of exposure, then it will not produce a permanent threshold shift in excess of 5 dB. The critical levels for TTS then define so-called "safe levels of noise," or "acoustic injury thresholds."

Critical levels are plotted in Figure 4, as well as the audibility and tolerance thresholds (*22*). Figure 4 can be taken as a range of likely acoustic injury and permanent hearing in humans. There are three

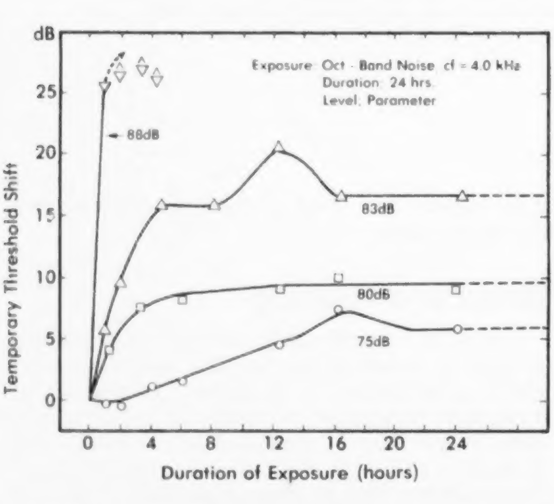

FIGURE 1. Temporary threshold shifts (TTS) as a function of the duration of exposure produced by an octave-band SPL of the noise centered at 4.0 kHz. The parameter is the octave-band SPL of the noise. Measurements of TTS were made about 4 min (TTS_4) after a subjects had been recovered from the noise. For the 88 dB exposure, some subjects had TTS's greater than 25 dB after 1-2 hr of the exposure. Therefore, the exposure was terminated (*7*).

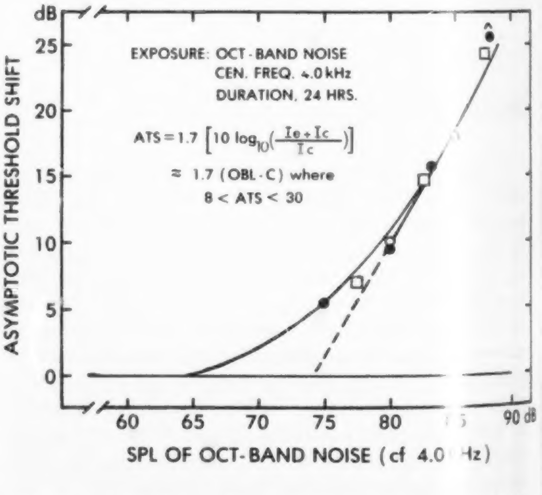

FIGURE 2. Relation between TTS at asymptote and the octave-band SPL of a noise centered at 4.0 kHz (7). Critical levels (*C*) are estimated by a straight line extrapolation as shown by the dotted line.

areas of interest in Figure 4. The region bounded by "injury thresholds" and the audibility curve represent sounds audible to humans which present no risk of injury to the ear regardless of the duration of exposure. The uppermost region (top of Fig. 4) represents sounds that present a high risk of injury. This region corresponds to so-called "discomfort thresholds" which are called discomfort (120 dB), tickle (125 dB), and pain (130 dB). Between the region of discomfort and the "injury thresholds" is the region where risk depends on the combined effects of level, duration, number of exposures, and individual differences. An increase in any of these exposure features increases risk, although the quantitative determination of the increased risk is the subject of much debate.

Individual differences in TTS and permanent threshold shifts (PTS) are not trivial (*7, 23*). Indeed, for a given noise exposure where the median TTS is 20 dB, individuals range from 5 to 35 dB. An explanation of individual differences, while not available currently, must surely include acoustic properties of the external middle, and inner ear, as well as details of the biological properties of the inner ear.

Field Studies: Permanent Effects. Noise-induced permanent threshold shifts (NIPTS) refers to permanent threshold shifts caused by exposure to industrial noise (corrected for age-related hearing loss). Most estimates of noise-induced permanent threshold shifts come from industrial field studies and are usually contaminated by a number of factors, including a chronic, temporary threshold shift component. The accuracy of these field studies is not universally accepted. Noise measurements, audiometric methods, control groups, and other factors are usually the focus of endless debate.

Regardless of these arguments, there are several excellent cross-sectional field studies in which the noise exposure was 8 hr per day (*2-6, 24*). A number of observations are common to these studies. At 4.0 kHz the median NIPTS increases rapidly during the first 10 years of exposure and then is asymptomatic or increases slightly between 20 and 50 years of exposure. The variability of NIPTS at 4.0 kHz as indicated by the semi-interquartile range reaches a maximum value of 12 to 20 dB after a few months or a few years and then decreases systematically to an asymptotic value of about 5 dB. It is quite possible that this asymptotic value of 5 dB is equal to the pre-exposure, semi-interquartile range (*2*). At 2.0 kHz the median NIPTS increases for about 30 years of exposure and then appears to

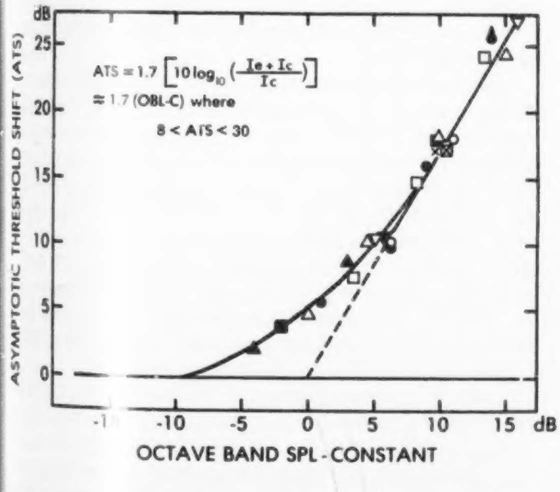

FIGURE 3. Relation between TTS at asymptote (at the test frequency of maximum shift) and the level of an octave-band noise minus a constant. Includes data from human, monkey, and chinchilla. The constant or critical level is dependent upon the center frequency of the noise and the species. For humans, the critical levels (*C* in the straight-line equation of Fig. 3) is 82 dB SPL at 0.5 and 1.0 kHz, 78 dB at 2.0 kHz and 74 dB at 4.0 kHz. For a wide-band noise, *C* is 78 dBA. For additional details see Mills et al. (*7*).

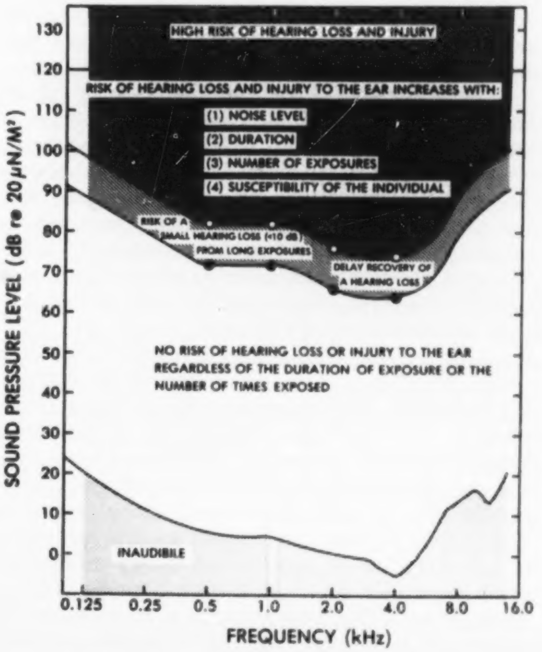

FIGURE 4. Range of human audibility categorized with respect to the likelihood of acoustic injury of the ear and noise-induced hearing loss (*26*).

reach an asymptote. Variability of NIPTS reaches a maximum after 20 years of exposure, remains constant between about 20 and 30 years of exposure, and then decreases and reaches an asymptotic value of about 5-7 dB after about 40 years of exposure.

The major difference between variability at 4.0 kHz and at 2.0 kHz appears to be in the temporal domain. That is, maximum semi-interquartile range at 2.0 kHz (16 to 17 dB) is nearly equal to the maximum at 4.0 kHz; however, the maximum at 2.0 kHz is reached after about 20 years of exposure, whereas the maximum at 4.0 kHz is reached after less than 5 years of exposure.

Of some significance is the possible relationship between the median value of NIPTS and the variability of NIPTS as indicated by the semi-interquartile range. The variability of NIPTS always reaches a maximum several years before the median NIPTS and at a time when the rate of change of the median is the greatest (or nearly so). Similarly, variability of NIPTS is smallest when the median NIPTS is largest and the rate of change of the median in minimal. In other words, the relation between the variability of NIPTS and median NIPTS is nearly identical to that observed in many dynamic systems where the variability of response varies directly with the rate of change of response. A complete explanation of individual differences is likely to require consideration of many acoustic, physiological and anatomical variables, as well as many nonauditory variables, such as diet and drugs.

Laboratory and Field Studies. Many of the laboratory studies of TTS have arisen from an interest in the problem of NIPTS. We make no effort to explain all the possible correlations between TTS and NIPTS. Rather, the effort here is to show the correspondence between results from laboratory studies of TTS and from field studies of NIPTS. Figure 5 shows threshold shifts plotted as a function of noise level, where noise level is corrected by subtracting a constant. Threshold shifts are TTS's from Figure 3 as well as additional TTS data from chinchillas (*10*, *18*, *19*). Other threshold shifts in Figure 5 are NIPTS's from field studies summarized by von Gierke and Johnson (*25*). Figure 5 has several features worthy of comment. One is that the empirical equations developed nearly 10 years ago for TTS in chinchillas can accurately describe TTS in man and monkeys, NIPTS (50th percentile) at 4.0 kHz after 10 years of exposure, and NIPTS (90th percentile) at 4.0 kHz after 10 or 30-40 years of exposure. The only correction required is the subtracted constant. That is, to equate the 90th percentile with the 50th percentile in NIPTS at 4.0 kHz after 10 years of exposure, noise level should be changed by 7.5 dB. To equate TTS with NIPTS (50th percentile, 10 year) noise level should be changed by 3 dB.

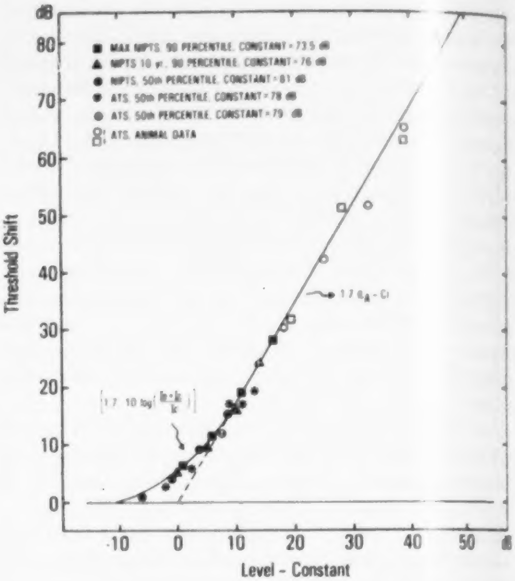

FIGURE 5. Relation between noise level and hearing loss. Data are from Figure 3 and field studies of noise-induced permanent threshold shifts in industrial workers (*25*). This figure links field studies with humans and laboratory studies with humans and animals (*58*).

The data shown in Figure 5 are supported also in a recent study (*24*) which shows an NIPTS of about 15 dB at 4.0 kHz after about 10 years of exposure to a noise with an A-weighted sound pressure level of 89 dB. TTS data from Figure 5 predict that NIPTS at 3.5-4.0 kHz would be less than 18.7 dB, and NIPTS data in figure 5 predict an NIPTS of 13 to 14 dB. The agreement between these data (*24*, *25*) and the different sets of NIPTS data shown in Figure 5 is striking. The predictions from TTS data can be considered excellent as well, particularly since the "error" of 3.7 dB is in the proper direction.

Currently, many persons are debating the merits of an 85 dBA noise for 8 hr versus 90 dBA for 8 hr. It is our belief that the audiometric effects of such exposure can be predicted from the data on Figure 5. Indeed, in our opinion the "85 dBA versus 90 dBA" controversy is clearly a social-economic-political issue which necessarily must be resolved by those who make social-economic-political decisions. In other words, data from additional experiments are not the solution to the 85 dBA versus 90 dBA controversy.

Mechanism of TTS and PTS. The mechanisms of TTS an PTS are not known. For moderate level exposure ne is inclined to think of a metabolic or biochemic bases, including the depletion of energy stores, as mbly of cell membranes, effects on proteins and ids of the cochlea, mechanically induced changes i the shape of the tectorial membrane, and vaso- nstriction within the cochlea. For high-level exp ure one is inclined toward a purely mechanic basis where in the input to the cochlea is intense en gh to drive the basilar membrane beyond its norm range of displacement. The attached structure organ of Corti) and unattached structures (Re ner's membrane) are thus "ruptured." The deter ination of the mechanism(s) of acoustic injury pr ably awaits the determination of the transducti n mechanism of the cochlea, and basic informatio on the biochemistry of the cochlea, including tion filaments of the stereocilia.

Noncontinuous Exposure

Whereas the data base is massive for the effects of continuous exposure to steady-state noise, the data base is less impressive for intermittent exposure to steady-state noise or intermittent exposure to noises with fluctuating levels. It is difficult to find occupational settings where the noise exposure can be clearly defined and measured accurately, and where there are a large number of employees who are employed for 20-40 years. Similarly, laboratory investigations must use humans (TTS) or animals, and the researcher is confronted with an almost endless list of variables. Moreover, because of regulatory efforts and their need for simplicity, there has been a fascination with the idea of a single-number correction factor. For example, one approach has been to equate noises with greatly varying temporal properties in terms of equal energy, equal pressure or compromises on equal energy or equal pressure. Thus, a steady-state noise exposure for 8 hr with a s und pressure level of 90 dBA is assumed to be equ to an exposure of 4 hr at 96 dBA, 95 dBA, 94 d A, or 93 dBA. In other words, there is great deb e and confusion regarding the exchange of the inte sity of the noise and the duration of the exposure. compromise is the "5 dB rule" currently use y OSHA and the Department of Labor.

Our th is is that a single-number correction factor wh h specifies a time-intensity trade-off in NIPTS or TS is doomed to be grossly incorrect at worst, or t best, to have a very restricted range of applic on. This thesis is based on available literature d the results of a current project in our laborator Our results (*26, 27*) indicate that a number t describe time/intensity trading ranges from 0 d to as large as 8 dB. The situation is complicated in part because of the ability of the ear to use quiet periods to recover from the noisy periods. In other words, rest periods as short as a few seconds may be useful in protecting the ear, particularly in those conditions where the noise is present for only a few minutes. It is perhaps because of these quiet periods that rock musicians and others similarly exposed do not have large hearing losses.

Impulse Noise

The acoustic impulses produced by handguns and rifles are the cause of mild-to-severe sensorineural hearing losses (*28*). High-frequency hearing losses are typical of persons exposed to acoustic impulses with peak sound pressure levels in excess of about 145-155 dB SPL. Among hunters and military personnel, PTS is usually largest in the left ear. The right ear (right handed shooter) receives less acoustic energy than the left because of the head-shadow effect.

The risk of NIPTS from acoustic impulses generally increases as the number of impulses increases, as the peak SPL of the impulses increases, and as the duration of each impulse increases. Also, specification of impulse duration is complicated, but apparently a critical factor. Detailed discussions of impulse noise and its effects on hearing are given elsewhere (*29*).

It is worth noting that laboratory investigations with human subjects are very difficult to do without placing the subjects at risk. An impulse at a given level and repetition rate may produce very little in the way of measurable effects. However, 10 dB increase in level may produce an unusually large shift. In other words, the range of levels available to an experimenter is small, and individual differences are large. It is disconcerting to note many anecdotal and clinical reports which show severe hearing loss after exposure to one impulse (acoustic trauma) or to a series of impulses (*29*). In fact, there are many reports documenting that acoustic impulses which had been innocuous on many previous occasions produce severe unilateral or bilateral PTS on a subsequent occasion.

The nature of the hearing loss produced by acoustic impulses is probably different from the nature of the hearing loss caused by continuous exposures to steady-state noise at moderate levels (for example, 90 dBA). That is, the displacement of the basilar membrane by an intense acoustic impulse may be sufficient to produce "ripping and tearing" effects. Purely mechanical injuries may be produced by acoustic impulses, whereas injuries produced by lower-level acoustic signals may be caused by metabolic, biochemical, or vascular effects.

Interactions

One acoustic event which is innocuous to the cochlea by itself can interact with another innocuous acoustic event to produce a large injury to the organ of Corti. The most dramatic example of "acoustic synergism" is the combined effects of noise and acoustic impulses (*30*). For example, a 157 dB impulse by itself or a noise of 95 dB produced no PTS or injury to the sensory cells, but the combined effect of the impulse and the noise was devastating. PTS ranged from 5 dB at 250 Hz, to 50 dB at 2.0 kHz and to 35 dB at 8.0 kHz. Outer hair cells were totally destroyed over a 7 mm region of the basilar membrane. Injury to inner hair cells was nearly as severe. These results have been documented in a series of experiments using other levels of impulses and continuous noise. There are, no doubt, other interactions between acoustic events which can either increase or decrease the risk of hearing loss. For example, a noise presented before an acoustic impulse can contract the stapedius muscle (acoustic reflex) (*31*). The impulse is then attenuated in the middle ear and the inner ear is thus protected. In the absence of the noise, the impulse travels through the middle ear before the reflex is activated (reflex latency 10 msec).

Exposure to Noise and Auditory Perceptual Deficits

Studies of auditory discrimination and reading skills of children living near freeways suggest deleterious environmental effects (*32*, *33*). In this study, where noise levels inside apartments ranged from 55 dBA to 75 dBA, the performance of 2nd to 5th grade children on standardized speech discrimination and reading tests were correlated (0.48, 0.53) with noise levels for children who had resided in the apartment complex for at least four years. Substantially more data are required to clarify all of the issues involved. Moreover, it remains unclear whether noise-related deficits in speech discrimination and reading are temporary and will be overcome by maturation and schooling, or are permanent. These data (*32*) may be the first to indicate strongly that the development of important skills such as listening and reading is being affected by environmental noise.

Aging

While it may or may not be legitimate to include aging as an environmental factor, the loss of hearing associated with aging is a major problem. It will become far more significant in the next decades; therefore, it is difficult to ignore in a discussion of causes of hearing loss.

The loss of hearing associated with aging is called presbyacusis. Presbyacusis has been subdivided into four categories: sensory, where the major pathology is at the outer and inner hair cells; neural, where the major pathology is indicated by large losses of spiral ganglion cells (cell body of Nerve VIII); metabolic, where major pathology is at the stria vascularis of the cochlea; and mechanical, where it is postulated that the elasticity or compliance of the basilar membrane and other structures is reduced. Of course, the CNS does not escape the effects of aging, nor does the sensitivity of other sensory systems, including gustatory, olfactory and ocular (*34*).

Auditory sensitivity for pure tones (audiogram) and other auditory behavior are affected in a manner that reflects to some extent the major pathology. For example, sensory and mechanical types of presbyacusis usually show moderate to severe losses in the high frequencies. In neural presbyacusis the audiogram may be normal or nearly so until greater than 50-80% of the spiral ganglion cells are missing. In cases of metabolic presbyacusis the hearing loss is moderate and affects all frequencies nearly equally.

While the quantitative facts of presbyacusis are important to clinical diagnosis and to patient management, they have significance as well to the problem of noise-induced hearing loss. That is, how much of a person's hearing loss is due to noises of everyday living (sociocusis); to previous infections, blows to the head, drugs, etc. (nosoacusis)? How do all of these causes of hearing loss interact and what is the nature of the interaction?

It is assumed that the effects of presbyacusis add linearly to the effects of occupational noise exposure. Moreover, presbyacusic control groups and occupational noise groups are assumed to have equal amounts of sociocusis and nosoacusis. Of course, these are extremely convenient assumptions which are necessary to permit the calculation of NIPTS. For group averages they may even have some accuracy. However, on an individual basis, gross errors probably occur. It is difficult to imagine that the additivity rules of neural presbyacusis is quantitatively identical to the additivity in mechanical presbyacusis.

The concept of presbyacusis has been altered by the results of studies completed in other cultures. Perhaps the most often quoted example is that of the Mabaan tribe of Africa (*35*). In this extremely quiet culture, the auditory thresholds of 80 or 90-year-old persons were approximately equal to those of 20-year-old persons in industrialized cities of North America and Europe. While some persons were quick to note the absence of noise, the authors pointed to the absence of cardiovascular disease, the absence of meat in the diet, the climate, the absence of stress, and the absence of peptic ulcers. It was concluded that hearing loss in old age is attributable not just to the effects of noise, drugs and aging, but to the cumulative effects of these factors plus diet and stress-lifestyle factors. It is equally important to note that in Mabaans who had moved to Egypt there was an unusually high incidence (for Mabaans) of hearing loss, cardiovascular disease and peptic ulcers. In other words, auditory skills are affected directly by definitive agents such as noise and drugs, and by lifestyle as well.

Ototoxic Drugs and Viral Infections

Ototoxicity from aminoglycoside antibiotics is well known (*36-40*). The aminoglycosides have a 2-deoxystreptamine as a central component. All are produced by the Streptomyces genus except the gentamycins, which are produced by a strain of *Micromonospora purpurea*. These antiboitics, which include kanamycin, gentamycin, neomycin, tobramycin, amikacin, sisomicin, netilmicin, and others, are bacteriocidal because they bind to proteins on the 30 S segment of the ribosome.

Aminglycoside antiboitics are indicated in the treatment of tuberculosis and serious gram negative infections with such bacteria as *E. coli*, Klebsiella, Proteus, *Pseudomonas aeruginosa*, and Serratia, for example (*36-40*). Aminoglycoside antibiotics are used to treat at least one million people in the U.S. annually (*40*), and perhaps as many as four million (*41*).

Ototoxicity from aminoglycoside antibiotics may be cochleotoxic and vestibulotoxic. Tobramycin and amikacin are considered more cochleotoxic, whereas streptomycin and gentamycin are considered more vestibulotoxic. The incidence of ototoxicity varies from 2% to 25% and perhaps as high as 55% (*40, 41*). In a prospective study of 38 courses of therapy in 113 patients (*40*), some significant associations with ototoxicity included high temperature elevated hematocrit, high creatinine clearance, poor condition of the patient and duration of therapy greater then 10 days. Serum levels, age, prior noise exposure and use of their ototoxic drugs were not found to be significantly correlated with the incidence of aminoglycoside ototoxicity.

Cochlear toxicity from aminoglycoside antibiotics affects the inner row of outer hair cells first, followed by the outer rows of hair cells, then by the inner hair cells (*40*). The pathology has also been localized to the stria vascularis, spiral ligament, and spiral prominence. Several theories of the mechanism of damage have been proposed, including direct damage to the hair cells and disruption of the metabolism in the stria vascularis and spiral ligament, which leads to changes in cationic differences of the perilymph and endolymph (*40*). The mechanism of vestibular damage remains poorly defined.

Hearing loss produced by aminoglycoside antibiotics ranges from minimal to severe. It usually starts at high frequencies and progresses to low frequencies. Efforts at early detection of otoxicity are sometimes only marginally successful at restricting the magnitude of the hearing loss. By the time reliable changes in the audiogram are detected, the degenerative process is underway and may continue for weeks or months after termination of the antibiotic.

Noise and some types of aminoglycosides interact synergistically in a most dramatic fashion (*41*). Noise exposure that produced minimal injury to the organ of Corti, and kanamycin administrations that also produced minimal injury acted synergistically and destroyed nearly 80% of the outer hair cells (*41*). No doubt, the aminoglycosides also interact with agents other than noise; however, the almost endless list of possible interactions is not well documented.

Whereas aminoglycosides can have severe effects on the organ of Corti and produce severe hearing loss, large doses of salicylates apparently produce only small (10-12 dB) hearing loss that is totally recoverable when the therapy is terminated. Moreover, there is little, if any, interaction between salicylates and noises of the impulse or steady-state variety (*44, 45*).

A large number of viral infections have been associated with sensorineural hearing losses. Rubella and mumps are the classic examples. Hearing loss with mumps is usually unilateral, whereas the loss with rubella is bilateral. In both cases the temporal bone pathology includes extensive degneration of sensory cells, stria vascularis, and tectorial membrane as well as the nerve supply (*46, 47*). Recently, the cytomegalovirus (CMV), a member of the herpes group, has been isolated from the perilymph and has been shown to cause congenital deafness. Inclusion-bearing cells have been observed in the epithelium of the utricular and saccular macula, Riessner's membrane and stria vascularis (*54*). It may possibly cause progressive hearing loss which starts at about two years of age (*48-51*). Severe middle ear infections are also associated with CMV (*51*).

Other viruses which have been implicated in both the gradual and sudden onset of sensorineural hearing loss include influenza, adenovirus, and herpes hominis (*52, 53*). Most viral infections of the inner ear are described as "endolymphatic labyrinthitis" with a pathology consisting of degeneration of the stria vascularis, organ of Corti, and tectorial membrane.

A large number of other factors are assumed to affect hearing. These include a long list of chemicals (*55*) and bacterial infections (*56*), as well as over 40 genetic and metabolic syndromes (*57*).

General Comments

It is our belief that the state of the art concerning the causes of hearing loss has advanced in the past twenty years and restrictions in the discussion of hearing and hearing loss often occur because of a lack of basic information. The study of the biochemistry of hearing is in its infancy, neural transmitters in the cochlea are not discovered, the transducer mechanism of the cochlea is still debated, and details of the neural "wiring" diagram of the cochlea are unresolved. Similarly, the function of the outer hair cells and the efferent auditory system is not agreed upon. When these basic issues are resolved, there ought to be significant gains made in the detection, diagnosis and treatment of sensorineural hearing loss.

The preparation of this manuscript was supported by a grant from NIEHS to the Medical University of South Carolina. Assistance in the preparation of the final document was provided by Janet Simmons.

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Environmental Health Perspectives
Vol. 44, pp. 129-133, 1982

Commentary

What Can Be Learned from Studying Asian Health Problems?

by Philip S. Chen, Jr.*

The National Institutes of Health, owing to its broad mandate to pursue and support acquisition of knowledge in biomedicine, is very much concerned with any investigations leading to better understanding of the causes and control of human diseases. One approach is to study health and illness in special subsets of the United States population and of populations in foreign countries.

In this paper, I shall touch briefly on a number of health problems and disease-oriented research topics looked at from the special perspective of Asian and Asian-American populations, hopefully to sensitize perceptions about the contributions that such studies can make to our total understanding of how best to achieve health and conquer disease.

The new goal of the World Health Organization (WHO) is "Health for all by the year 2000." "The aim is to enable all people of the world to attain an acceptable level of health and to lead a socially and economically productive life." (*1*).

Population

Asia, with just over 20% of the world's land area, has over 55% of the world's population. The two most populous countries, China and India, alone account for over 40% of the world's population, and Indonesia is the fifth most populous nation, Japan is seventh, followed by Bangladesh and Pakistan.

An important factor in determining the health of a nation's population is the level of economic development, which in turn is related to the rate of population growth. Growth in population puts enormous pressures on many aspects of a nation's economy. The larger the number of infants and young children who need to be fed and cared for, the greater the number of those who will live in poverty, be uneducated, and underfed.

The annual rates of population growth range from 0.4% in northern Europe to 1.0% in North America and the U.S.S.R. to 1.2% in Japan and 1.6% in China, to 2.6% in South Asia, and to 3.3% in Middle America. The world average is 1.9%.

Control of population growth rates will correlate with improvements in health, and a number of Asian countries are continuing to struggle with this significant aspect affecting their national futures.

Malnutrition

Recent estimates by the Food and Agriculture Organization (FAO) of the United Nations suggest that about 450 million people do not receive enough food. The distribution of malnutrition is very uneven around the world, even within a region such as Asia (*1*). In high-income countries with low rates of population growth, the general problem is obesity due to overeating (i.e., malnutrition of affluence). These countries include Japan and Oceania, as well as those in North America and Europe. In middle-income developing countries, some of which are in Asia, there are high rates of population growth, poor income and land distribution, resulting in food deprivation for many. The higher income portion of the population may also suffer from malnutrition of affluence. The poorest countries, including those in Asia and the middle of Africa, are those in which hunger and malnutrition are most severe. An estimated one-third to one-half of their inhabitants are victims of malnutrition.

China and other Asian countries with planned economies are in a special category. Although per capita GNP is in the lower range, most observers report that overt malnutrition is not common. Thus it is possible for a nation to improve the nutritional status of its populace by devoting long-range attention to this issue. Health research *per se* is not particularly competent to contribute to the solutions of these kinds of malnutrition problems.

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Delivery of Health Care

In Asia, as is true even of a modern industrialized nation such as the United States, there are considerable variations in the quality of health care available to the general population. One general indicator that has been used is the ratio of physicians to the number of inhabitants. In the United States this ratio is about 168 per 100,000 population, which is roughly double the physician:population ratio worldwide of 80.8 per 100,000. In Asia, the number per 100,000 population ranges from a surprising high of 208 in Mongolia, to 26 and 27 in Pakistan and India. The comparable figures for Japan and China are 118.3 and 38.4, respectively, based on the latest World Health Organization figures for 1980. At the low end are Thailand with 12.1 and Indonesia with only 7 (*2*).

These figures may be misleading, in the sense that not all health care is delivered by physicians. For example, we have all heard of the great contributions of the "barefoot doctors" in the People's Republic of China. These paraprofessionals, as well as the more highly educated physicians, are trained in the use of traditional Chinese medicine as well as in Western medical knowledge. Much publicity had been given to the use of acupuncture following former President Nixon's trip to China, and his personal physician, Dr. Walter R. Tkach, encouraged the NIH to support research on acupuncture. At that time, I was working for the National Institute of General Medical Sciences, and Dr. DeWitt Stetten, the then Director of NIGMS, was asked by the NIH Director, Dr. Robert Q. Marston, to initiate an acupuncture research program. The NIGMS convened international workshops, and a review committee was established within the Division of Research Grants to evaluate grant applications in acupuncture. An initial flurry of research began, coincident with the opening of many acupuncture clinics scattered around this country. In the last half dozen years or so, however, interest in acupuncture has diminished and stabilized. It is generally accepted that acupuncture can exert an analgesic effect, mediated perhaps through some mechanism involving the endorphins. The claims for therapeutic effects of acupuncture in the cure of a wide variety of diseases are generally not supported by reliable research evidence.

An example of the contribution of traditional Chinese medicine to Western medical knowledge is the discovery of ephedrine through scientific studies of an old Chinese herbal concoction. Several decades ago, the Chinese-American pharmacologist, Dr. K. K. Chen, performed, in this country, the work which led to the acceptance of ephedrine as a standard pharmaceutical agent.

I might add at this point a brief comment about the reported remarkable improvement in health care in the People's Republic of China and the manner in which it was attained. Certain lessons can be learned from this which have applicability to other Asian countries as well as to the United States.

Soon after founding of the People's Republic of China, Chairman Mao issued four cardinal principles as guidelines for medical and health work in China. One was to stress prevention, another was to unite the practitioners of Chinese traditional medicine and of Western medicine. Today it is accepted that China has advanced considerably: in its health delivery system, using barefoot doctors, health aides, and midwives; in its medical education system; in its production of pharmaceuticals, medical instruments and apparatus; in its sanitation efforts, resulting in eradication or control of smallpox, cholera, plague, and venereal diseases; in its family planning programs; and in its integration of traditional medicine with modern Western practices.

Cancer

In a monumental study recently completed in the People's Republic of China, data on cancer mortality over the three-year interval 1973-1975 were collected among a population of 840 million Chinese using a cadre of approximately 600,000 health workers. Results were issued in a Chinese publication entitled *Atlas of Cancer Mortality for the People's Republic of China* (*3*). The data are most dramatically displayed in colored maps showing mortality rates for various cancers by county. About 700,000 deaths per year were due to cancer.

Some dramatic differences between the Chinese and the U.S. data can be highlighted. In the United States, the leading cause of cancer mortality is lung cancer in men and breast cancer in women. In contrast, the leading cause of cancer mortality in both men and women in China is stomach cancer, followed by cancers of esophagus and liver. In the United States lung and breast cancer are followed by colon and rectal cancer.

Overall cancer mortality in China is higher in urban areas, in males, and in certain ethnic minorities. Among children under 15 years of age, cancer is the cause of only 0.8 percent of deaths. The overall statistics placed China lower in cancer mortality than most industrialized nations but higher than many other nations in Africa, Asia, and Latin America.

Certain variations within the country are very interestin from a research standpoint. For example, esoph geal cancer varies markedly with geographical reas and there are six areas in China with unus lly high rates. For example, in Linxian, Hanan pr vince, esophogeal cancer rates may be the highe in the world, with one death in six in this count attributed to esophageal cancer.

The co ty maps of China show that there are high rates f nasopharyngeal carcinoma in southern China. S eral factors are associated with this cancer. F example, antibodies against Epstein-Barr viru end to be elevated in patients with this disease a can be used as an assay in screening programs Genetic factors may also play a role, because t re are higher rates among Cantonese who migr ed to that region several generations ago. Also here are high incidences of liver cancer on the so theastern coastal provinces and in an area of no theastern China, which may be linked with liver disease caused by viral hepatitis B, with ingestion as a contaminant in the diet of aflatoxin, known as a potent liver carcinogen, and with possible exposure to hepatotoxic pesticides.

Cancers of the lung and bladder are found to be more common in major urban areas, such as Shanghai, Tiensin and Beijing. Contributing factors might be an increased level of cigarette smoking or of industrial pollutants and the use of coal-burning stoves in unventilated homes. Dr. Robert Miller of the National Cancer Institute reported on a valuable data resource in Shanghai—a tumor registry containing information on incidence and mortality going back to the 1960s (*4*). By comparing cancer incidence in Shanghai with that for Chinese-Americans and other ethnic groups in the United States, epidemiologists may be able to gain useful knowledge concerning possible etiologic factors. The Shanghai data show that cancer of the prostate and Ewing's sarcoma of bone are rare, whereas stomach cancer is the most frequent neoplasia.

The Na ional Cancer Institute has sponsored a number o xchange visits of scientists between our country a d China. Several NCI epidemiologists have spe time traveling in or working in China. Reports the special clustering of cancer in domestic nimals, such as pigs, chickens, and ducks, ha provided very useful clues in research on the et ogy of human cancer.

Dr. Ro ert Miller has also worked in Japan and related t interesting discovery of a very high incidence f primary respiratory tract cancers in Japanese who worked in a mustard gas factory during W ld War II. The latest statistics indicated that 73 o 500 workers had fallen victim to such cancers.

Genetic Diseases

A recent visitor to Beijing reported to the WHO on quantitative differences between the Chinese population and the general U.S. population with respect to several genetic diseases. For example, cystic fibrosis and several leukodystrophies were seen relatively infrequently among the Chinese. However, other genetic diseases are observed relatively frequently, such as Down's syndrome and other chromosomal defects, Huntington's chorea, phenylketonuria, homocystinuria, Duchenne's muscular dystrophy, and even Tay-Sachs disease in a certain province (Hanan). The Tay-Sachs gene may have been introduced into the latter geographic area during the 13th century, when it was said that Jewish traders had lived in Kai-feng City, marrying Chinese spouses.

The unavailability of special diets such as low-phenylalanine for patients with phenylketonuria is a problem. U.S. medical scientists, through visits to China, are helping to provide advice with respect to better diagnostic methods and treatment modalities for genetic diseases. As the general level of technology improves in the study of genetic diseases, there will undoubtedly be significant findings that will become evident owing to the very large and yet mostly homogeneous population in a country as large as China. The same can be said of other Asian countries.

Infectious Diseases

Most Asian populations do not differ from those in Western countries in their basic reactions to disease agents. However, a number of infectious diseases remain very serious problems in particular Asian populations, in contrast to the U.S. where they are rarely or never seen.

Parasitic Diseases

Malaria continues to be of global impact, especially in South Asia. In 1976 there were over 8 million cases. One of the serious problems today is falciparum malaria, resistant to chemotherapy. Considerable research efforts are aimed at immunologic approaches, i.e., the development of a vaccine. There is evidence that vaccines might be able to be produced from practically all stages of the parasite in man (sporozoites, schizonts, gametocytes, merozoites). One intriguing approach is to attempt to interrupt transmission, in effect by "vaccinating" the mosquito.

Transmitted by snails as a vector, Schistosomiasis has increased in incidence as a result of large-scale water impoundments and artificial man-made lakes. Some 200 million people are infected. Research is aimed at improved chemotherapeutic agents. The most recent drug is called Praziquantel, said to be especially useful in treating *S. hematobium*.

Acute Diarrheal Diseases

Diarrheal diseases are major killers of children, causing in excess of 5 million deaths annually. Rotavirus and enterotoxic *E. coli* as well as cholera are the major culprits.

Tuberculosis

Tuberculosis, once a public health problem in Western nations, accounts for 4th place among causes of death in Southeast Asia. There are 3.5 million new cases annually with 0.5 million deaths.

Leprosy

Four million individuals are infected with leprosy in Southeast Asia, mostly in India.

Infectious Disease Research

Research on these "tropical" and other diseases has been assisted by planned efforts in which NIH has played a significant role. The United States–Japan Cooperative Science Program is now in its 16th year. This program is coordinated for the U.S. side by the National Institute of Allergy and Infectious Diseases (5).

Certain laboratories located in Asia have received considerable encouragement and sponsorship by Western nations. The International Center for Diarrheal Diseases Research (ICDDR) in Dacca, Bangladesh, and also the International Center for Medical Research in Ching-Mai, Thailand, are sites where NIH staff have actively participated in many research studies. The National Institute of Allergy and Infectious Diseases is currently conducting field trials of hepatitis B vaccine for both its primary effect in protecting a mother from hepatitis as well as a possible secondary effect to protect offspring from later developing liver cancer. This is obviously a very long-term clinical trial but one with enormous implications, both for infectious disease and in cancer prevention.

Radiation Exposure

One truly unique opportunity available only in Asia is the ability to study the long-term effects of radiation exposure in the atomic bomb survivors of Hiroshima and Nagasaki, Japan. Nowhere else in the world is there such a population. A number of NIH staff, primarily from the NCI, but also from NIEHS and other institutes, have worked at the Radiation Effects Research Foundation (RERF) and its predecessor the Atomic Bomb Casualty Commission (ABCC).

The evidence that whole body irradiation can produce leukemia was firmly established by observations of the occurrence of acute and of chronic leukemia, both of which began to increase several years after the atomic bombs were dropped, peaking some 6 years later, but with continued higher incidence long afterwards. Prior to such direct observations on a large exposed population, the evidence for radiation-induced leukemia was based on individual case reports of early radiation workers or of radiologists, and experimental studies of the effects of x-rays in mice.

Despite the fact that their populations greatly exceed ours, the Asian countries have many of the same problems. Their leading causes of death are the same as ours: heart disease, malignant neoplasms and accidents. They are beset by the same behavioral factors as we are. Smoking is well recognized as a leading environmental health hazard. Just consider a few statistics on the number of cigarettes consumed by persons aged 15 or older in 1973: In the U.S. it was 3850; in Japan, 3240; in the U.K., 3230; in Hong Kong, 2780; in Singapore, 2490; in Pakistan, 760; and in India, 170. Clearly, several Asian countries are approaching the U.S. in a high cigarette consumption, and it can be expected that lung cancer, emphysema, heart disease, etc. will increase as a result. Indeed China, India, Indonesia, and the Republic of Korea are among the world's top 10 tobacco-producing countries.

In closing, the participation by Asian scientists in the total biomedical research effort should be noted. Many researchers and research support staff at the NIH as well as around the country are from an Asian heritage. Currently, the largest number of participants in the NIH Visiting Program (comprising Visiting Fellows, Visiting Associates, and Visting Scientists) comes from Japan. Many other Asian countries are represented as well. Since the commencement of diplomatic relations with the People's Republic of China, an increasing flow of research trainees, primarily Visiting Fellows, have come from the PRC. The "cultural revolution" unfortunately had caused a massive disruption in the research and educational system in China; as a consequence, its biomedical researchers were not able to keep pace with the rapid progress in science. The National Institutes of Health is contributing to the upgrading of biomedical research capability in that nation by providing many training opportunities for PRC scientists ithin the intramural NIH laboratories and clinic

Health esearch and efforts to improve health care are aceful activities which can greatly contribute to man well-being. They are noble endeavors of whi mankind can be proud. Such studies of and by A ns and Asian-Americans help to meet our own a irations for health as well as the overall global go set by the World Health Organization.

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Contributed Articles

Environmental Health Perspectives
Vol. 44, pp. 137-146, 1982

Toxicology of Chloral Hydrate in the Mouse

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Chloral hydrate has been found in our drinking water supplies at levels up to 5 μg/l. The purpose of this study was to evalute the acute and subchronic toxicology of chloral hydrate in the random-bred CD-1 mouse, to provide data for risk assessment. The acute oral LD_{50} of this compound was 1442 and 1265 mg/kg in male and female mice, respectively. Acute toxicity appeared to be related to depression of the central nervous system. Fourteen-day exposure by gavage in male mice at doses 1/10 and 1/100 the LD_{50} caused an increase in liver weight and a decrease in spleen weight at the highest dose level. Based on the data derived from 14 days of exposure, a 90-day study was performed. The compound was delivered via the drinking water; levels of the compound delivered per day were equivalent to those dosed in the 14-day study. The target organ in both sexes appeared to be the liver, with the males most affected. Male mice demonstrated a dose-related hepatomegaly accompanied by significant changes in serum chemistries and hepatic microsomal parameters. The females did not demonstrate the hepatomegaly observed in males, but did show alterations in hepatic microsomal parameters. No other significant toxicological changes were observed in either sex following 90 days of exposure.

Introduction

Chloral hydrate has been identified as a water contaminant in several of the water supplies surveyed by the EPA (*1*). Its presence in the drinking water stems from its industrial use in the preparation of polyurethanes. After its introduction as a therapeutic agent in 1869, it was commonly used as a hypnotic until well into the twentieth century, when it was largely supplanted by other drugs. However, it is still used as a sedative in doses usually ranging from 0.5 to 1.0 g, but sometimes as high as 2 g (*2*). Aside from this, exposure to chloral hydrate may be secondary to exposure to trichloroethylene, another water contaminant which is transformed into chloral hydrate during its metabolism (*3*).

While the toxic effects of chloral hydrate as a therapeutic agent have been well documented, the effects of long-term oral exposure at lower levels are not known. The purpose of this study was to describe the toxicity of chloral hydrate administered in the drinking water.

Methods

Animals and Housing

Random-bred CD-1 mice were obtained from Charles River Breeding Laboratories, Wilmington, MA. They were housed four per cage in plastic shoebox cages containing sawdust bedding (PWI

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Hardwood Sawdust, Lowville, N.Y.), and maintained on Agway Lab Chow *ad libitum*. The animal room was maintained at 21–24°C and 40–60% relative humidity. The light/dark cycle was maintained on 12-hr intervals. All mice were quarantined for one week prior to use and individually identified by earpunching.

For acute toxicity studies, 6-week-old male and female mice were used. For the 14-day study, 5-week-old mice were purchased, and exposure began at six weeks of age. For the 90-day study, 3-week-old mice were purchased, and exposure began at 4 weeks of age.

Chemical

Chloral (U.S.P. crystalline, J. T. Baker Co., Phillipsburg, N.J. 08865, lot #925086) was used in these investigations and the structure confirmed by infrared spectroscopy. The manufacturer stated purity was 99+%.

Chemical Administration

Solutions of chloral hydrate were prepared fresh daily in deionized water for acute and 14-day studies, and the appropriate concentrations were administered by gavage in a volume of 0.01 ml/g body weight to achieve the desired dose. The doses used in the 14-day study were 14.4 and 144 mg/kg. For the 90-day study, chloral hydrate was diluted in deionized water to concentrations of 0.07 and 0.7 mg/ml and administered as the drinking water. Drinking water solutions were maintained at room temperature in amber-colored bottles fitted with sipper spouts and were changed twice weekly. Less than 10% of the chloral hydrate was lost during the 3-4 days in water bottles. The analytical method employed GLC and head space analysis.

For the 90-day study, fluid consumption was calculated by weighing the water bottles at the time solutions were initially placed in them, then weighing the bottles once again before solutions were changed to determine the amount consumed over the 3- or 4-day period. Twelve cages of control mice and eight cages of each treatment group per sex were used to estimate fluid consumption. Chemical consumption was calculated from fluid consumption and is reported as grams of chemical/mouse/day and grams of chemical/kg body weight/day.

Acute Toxicity

Chloral hydrate was administered to male and female mice by an 18-gauge stainless steel stomach tube 18 hr after fasting from food. Seven doses were administered: 0, 300, 600, 900, 1200, 1500 and 1800 mg/kg. Eight mice of each sex were in each dose group. Following gavage, the mice were observed for behavioral and toxicological effects continuously for 4 hr and then twice daily for 14 days. Mice dying during the 14-day experimental period were necropsied, and gross pathological changes were described. Mice surviving the 14-day observation period were sacrificed and gross pathology was described. Log probit analysis ([illegible]) was used to determine the LD_{50}, 95% confidence limits and the LOG probit slope.

Necropsy

Animals were anesthetized with chloroform, and blood was collected immediately by cardiac puncture into 3.2% sodium citrate to be used for hematological evaluation. The organs (brain, liver, spleen, lungs, thymus, kidneys, and testes) were removed, trimmed, weighed, and gross pathological examinations were performed on all mice. Livers and spleens were used in further studies.

Urinalysis

Urine was collected at sacrifice and analyzed using Labstix reagent strips from Ames Co. (Elkhart, Ind.).

Hematology

Blood samples were taken as described above. Leukocyte, erythrocyte, and platelet counts were performed in a Coulter counter, Model ZBI. Hematocrits were performed with microhematocrit equipment, and hemoglobins were determined as cyanomethemoglobin. Leukocyte differentials were evaluated by the standard Wright's-Giemsa staining procedure. Bone marrow cells were flushed from the femur into α-Minimal Essential Medium (Gibco) with 5% fetal calf serum and enumerated on the Coulter counter.

Coagulation

The plasma from the blood samples was assayed for extrinsic activity by prothrombin time and for intrinsic activity by activated partial thromboplastin time (APTT). Reagents were obtained from General Diagnostics (Morris Plains, N.J.). Fibrinogen levels were determined by the kinetic method, using reagents from Dade Diagnostics, Inc. (Miami, Fla.)

Serum and Liver Chemistries

For serum chemistries, additional blood samples were drawn by cardiac puncture from animals involved in a study on humoral immunity (*5*) and allowed to clot. The sera from these samples were loaded onto the Abbott Bichromatic Analyzer, Model 100, and processed according to the procedures described in the Abbott Operator's Manual. Liver non-protein sulfhydryl levels were determined as described previously (*6*).

Preparation and Assay of Microsomal Parameters

Livers were removed, weighed, rinsed and homogenized at 4°C in 4 volumes of 0.15*M* KCl containing 0.01*M* potassium phosphate buffer, pH 7.4. Individual homogenates were prepared for each mouse. The homgenate was centrifuged at 9000 *g* for 20 min and the supernatant fluid recentrifuged at 100,000 *g* for 1 hr in a Beckman Model L5-50 ultracentrifuge. The microsomal pellet was resuspended in 0.05*M* potassium phosphate buffer, pH 7.4 (0.5 g of liver/ml), and samples were taken for immediate study.

Microsomal protein was assayed by the method of Lowry (*7*). The cytochrome P-450 content was determined from the CO difference spectrum, in dithionite reduced microsomes by using an extinction coefficient (*8*) of 91 cm^{-1} mM^{-1}. Aminopyrine *N*-demethylase activity was determined by measuring formaldehyde production by the Nash reaction as described by Cochin and Axelrod (*9*). Aniline hydroxylase activity was measured as *p*-aminophenol production (*10*).

Results

Acute Toxicity

The acute toxicity data for chloral hydrate based upon the 14-day observation period are shown in Table 1. The LD_{50} for the females was 1265 mg/kg and for males 1442 mg/kg. The slope of the dose-response curves was 32.6 for females and 30.6 for males. Within 10 min of gavage, the mice became sedated at the low doses, while at the intermediate and higher doses, the animals became lethargic and exhibited loss of righting reflex. Respiration was markedly inhibited with the higher dosages; this inhibition appeared to be the immediate cause of death. Animals necropsied after death showed gastric hypermia, but were otherwise unremarkable. Most deaths occurred within 4 hr at the highest dose. At lower dosages, some deaths occurred after 4 hr, with all deaths occurring within 24 hr.

Statistical Evaluation

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's *T*-test was performed (*11*). Values which differ from vehicle control at $p < 0.05$ are noted in the tables. Each of the values in the tables is given as the mean ± standard error (SE) of the mean.

Fourteen-Day Range Finding Study

Chloral hydrate solutions were administered to male mice by daily gavage for 14 consecutive days at doses of 14.4 and 144 mg/kg, which were 1/100 and 1/10 the LD_{50}.

Table 1. Acute toxicity of chloral hydrate in CD-1 mice.

Exposure group, mg/kg[a]	Sex	Number dead	LD_{50}, mg/kg[b]	Log probit slope
0	M	0	1442	30.6
300	M	0	(1290–1605[b])	
600	M	0		
900	M	0		
200	M	1		
500	M	4		
800	M	8		
0	F	0	1265	32.6
300	F	0	(1097–1405)	
600	F	0		
900	F	0		
200	F	3		
500	F	7		
800	F	8		

[a] There were eight animals in each dose group, with a total of 56 mice per sex.
[b] The numbers in parentheses represent the 95% confidence limits.

Table 2. Body weights of CD-1 male mice over 14 days of gavaging with chloral hydrate.

Exposure group	Day 1		Day 8		Day 15	
	Number of mice	BW, g[a]	Number of mice	BW, g[a]	Number of mice	BW, g[a]
Deionized water	68	25.1 ± 0.2	68	27.5 ± 0.3	66	.3 ± 0.3
14.4 mg/kg	60	25.2 ± 0.2	60	27.9 ± 0.3	59	.7 ± 0.3
144 mg/kg	60	25.5 ± 0.2	60	27.8 ± 0.3	59	.5 ± 0.3

[a]Body weight expressed as mean ± SE.
[b]Day 15 was 24 hr after the last day of gavage.

No significant changes in body weight were seen between the control and chloral hydrate-exposed mice over the 14-day exposure period (Table 2). One mouse of 60 died in the second week of gavaging in each chloral hydrate exposure group. These deaths were probably not compound-related since two mice of 68 died in the control group.

Of the general toxicological parameters measured, it appears that the livers and spleens of mice gavaged at 144 mg/kg were primarily affected. When expressed as percentage of body weight, the liver size increased 18%, while the spleen size decreased 27% compared to the vehicle control (Table 3). These changes were also seen when the organ weights were expressed in milligrams or as organ to brain ratios. Mice that received the lower dose (14.4 mg/kg) also demonstrated the same findings but not at values significantly different from control at $p < 0.05$.

Hematological and coagulation values were within control values for both dose groups (Table 4).

Serum glutamic pyruvic transaminase activity and blood urea nitrogen levels were within control

Table 3. Body and organ weights of CD-1 male mice exposed for 14 days to chloral hydrate.[a]

Exposure group	Body weight, g	Organ	Weight, mg	% of body weight	Organ/brain
Deionized water	31.0 ± 0.6	Brain	456 ± 7	1.48 ± 0.03	—
		Liver	1923 ± 96	6.18 ± 0.22	4.23 ± 0.22
		Spleen	227 ± 18	0.73 ± 0.06	0.50 ± 0.04
		Lungs	230 ± 5	0.74 ± 0.02	0.51 ± 0.02
		Thymus	89 ± 5	0.29 ± 0.01	0.19 ± 0.01
		Kidneys	597 ± 15	1.93 ± 0.05	1.31 ± 0.02
		Testes	229 ± 8	0.74 ± 0.02	0.50 ± 0.01
14.4 mg/kg	31.6 ± 0.6	Brain	446 ± 9	1.41 ± 0.04	—
		Liver	2109 ± 53	6.66 ± 0.12	4.75 ± 0.15
		Spleen	197 ± 14	0.62 ± 0.12	0.44 ± 0.03
		Lungs	228 ± 5	0.72 ± 0.02	0.51 ± 0.01
		Thymus	98 ± 7	0.31 ± 0.02	0.22 ± 0.02
		Kidneys	549 ± 24	1.73 ± 0.06[b]	1.24 ± 0.06
		Testes	240 ± 7	0.76 ± 0.02	0.54 ± 0.02
144 mg/kg	31.0 ± 0.9	Brain	441 ± 9	1.43 ± 0.04	—
		Liver	2255 ± 74[b]	7.30 ± 0.17[b]	5.11 ± 0.14[b]
		Spleen	166 ± 9[b]	0.54 ± 0.02[b]	0.38 ± 0.02[b]
		Lungs	223 ± 6	0.72 ± 0.02	0.51 ± 0.01
		Thymus	82 ± 6	0.26 ± 0.02	0.19 ± 0.01
		Kidneys	563 ± 21	1.82 ± 0.06	1.28 ± 0.04
		Testes	229 ± 5	0.74 ± 0.02	0.52 ± 0.02

[a]Values represent the mean ± SE derived from 11-12 mice/group.
[b]Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

Table 4. Hematological and coagulation values for CD-1 male mice exposed for 14 days to chloral hydrate.[a]

Exposure group	Hematocrit, %	Hemoglobin, g-%	Leukocytes × 10^{-3}/mm^3	Fibrinogen, mg-%	Prothrombin time, sec
Deionized water	40 ± 1	12.9 ± 0.3	6.79 ± 0.50	284 ± 7	8.2 ± 0.1
14.4 mg/kg	39 ± 0.5	12.4 ± 0.2	5.84 ± 0.36	284 ± 15	8.1 ± 0.1
144 mg/kg	41 ± 1	12.9 ± 0.2	6.36 ± 0.48	272 ± 7	8.4 ± 0.1

[a]Values represent the mean ± SE derived from 11-12 mice/group.

Table 5. Serum enzyme and urea nitrogen levels in CD-1 male mice exposed for 14 days to chloral hydrate.[a]

Exposure group	LDH, IU/l.[b]	SGPT, IU/l.[c]	BUN, mg-%[d]
Deionized water	908 ± 72	53.5 ± 5.3	27.8 ± 0.9
14.4 mg/kg	923 ± 46	78.2 ± 10.9	29.2 ± 1.5
144 mg/kg	727 ± 44[e]	73.5 ± 10.7	27.6 ± 1.0

[a] Values represent the mean ± SE derived from 10-12 mice/group.
[b] LDH = lactic dehydrogenase.
[c] SGPT = serum glutamic pyruvic transaminase.
[d] BUN = blood urea nitrogen.
[e] Significantly different from control at $p < 0.05$ as determined by Dunnett's T-test.

ranges. However, lactic dehydrogenase activity was depressed [illegible]0% compared to control in mice gavaged at 144 mg/kg (Table 5). This is difficult to interpret, since most reported abnormalities exhibit elevations, and not depressions.

Ninety-Day Study

Since the toxicity observed in the range-finding study was minimal, the concentrations of the chemicals administered in the 90-day drinking water study were calculated to deliver the same doses as the 14-day study. These concentrations were 0.07 and 0.7 mg chloral hydrate/ml. A group receiving deionized water served as controls. There were 260 mice of each sex in the control group and 140 of each sex in each treatment group. Forty-eight mice of each sex in the control group and 32 mice of each sex in the treatment groups were randomly selected and their body weight and fluid consumption were recorded twice weekly. The time-weighted average for chloral hydrate intake was 18 and 173 mg/kg for the females and 16 and 160 mg/kg for the males at 0.07 and 0.7 mg/ml, respectively. Table 6 shows the 30-day breakdown of daily fluid and chemical consumption per mouse and per kilogram. For consistency, all the toxicological data are presented according to concentration of chloral hydrate in the drinking water.

The growth curves for mice exposed to chloral hydrate are shown in Figures 1 and 2. Male mice exposed to chloral hydrate showed a dose-dependent increase in body weight (Fig. 1) over the 90-day period. Weight gains over this period were 14.1 for controls, 15.4 for the 0.07 mg/ml group, and 17.1 for the 0.7 mg/ml group. These weight increases were confirmed in mice used for gross pathology, which had increases above control of 8% for the 0.07 mg/ml group and 12% for the 0.7 mg/ml group (Table 7). There was no apparent effect of chloral hydrate on the growth rate of females (Figure 2). The average changes in female body weight over the 90-day period was 10.5 g for controls, 9.7 g for the 0.07 mg/ml group, and 11.7 g for the 0.7 mg/ml group. However, in half of the female mice (those used for gross pathology), the ones exposed to the highest concentration of chloral hydrate were 11% heavier than the controls (Table 7).

There was no compound-related gross pathology seen at the time of necropsy in either male or female mice. However, liver weights, as percentage of body weight, were increased in male mice receiving 0.07 mg/ml (12%) and 0.7 mg/ml (20%) (Table 7). This increase in liver size was also seen when weights were expressed on a milligram basis. No significant hepatomegaly occurred in the females. Spleen, thymus, kidney, and testes weights were not altered by exposure to chloral hydrate. There was a slight decrease in the lung weights of male mice, but the effect was not dose dependent and was significant only when the weights were expressed as percentage of body weight. This trend was also

Table 6. Time-weighted averages of consumption of fluid and chloral hydrate by CD-1 mice in a subchronic 90-day study.[a]

Sex	Concentration, mg/ml	Day 0–30		Days 31–60		Days 61–90		Days 0–90		
		ml/kg/day	mg/kg/day	ml/kg/day	mg/kg/day	ml/kg/day	mg/kg/day	ml/kg/day	mg/kg/day	ml/mouse/day
M	0	351 ± 10	0	217 ± 6	0	256 ± 10	0	273 ± 10	0	8.5 ± 0.3
M	0.07	276 ± 7	19.3 ± 0.5	201 ± 5	14.1 ± 0.3	191 ± 4	13.4 ± 0.3	225 ± 9	15.7 ± 0.6	7.3 ± 0.3
M	0.7	347 ± 8	242.9 ± 5.5	178 ± 3	124.9 ± 3.5	183 ± 3	128.0 ± 2.2	228 ± 5	159.8 ± 3.8	7.7 ± 0.2
F	0	327 ± 6	0	234 ± 6	0	194 ± 6	0	256 ± 9	0	7.1 ± 0.3
F	0.07	319 ± 9	22.3 ± 7	253 ± 7	17.7 ± 0.5	232 ± 7	16.3 ± 0.5	261 ± 11	18.2 ± 0.8	7.1 ± 0.3
F	0.7	329 ± 10	230.3 ± 6.8	210 ± 5	147.0 ± 3.3	203 ± 5	142.2 ± 3.7	248 ± 7	173.4 ± 5.2	6.9 ± 0.2

[a] Values represent the mean ± SE derived from 48 mice in the deionized water group and 32 mice in the other groups.

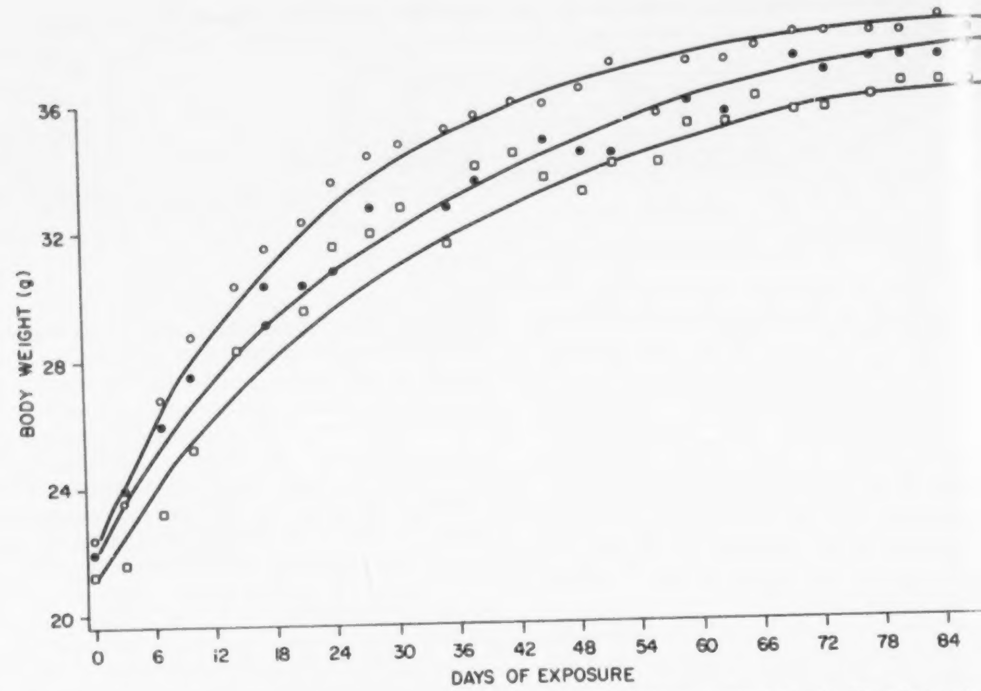

FIGURE 1. Growth chart of male CD-1 mice receiving chloral hydrate in the drinking water for 3 months. The mice receiv following: (□) deionized water (48 mice) (0 chloral hydrate); (●) chloral hydrate (32 mice), 0.07 mg/ml (16 mg/kg/day hydrate (32 mice), 0.7 mg/ml (160 mg/kg/day).

Table 7. Body and organ weights of CD-1 mice exposed to chloral hydrate in the drinking water for 3 mon

	Males			Females	
Exposure Group	Deionized water (n = 21)	0.07 mg/ml (n = 15)	0.7 mg/ml (n = 16)	Deionized water (n = 22)	0.07 mg/ml (n = 13)
Body weight, g	33.9 ± 1.1	36.8 ± 1.0[b]	38.0 ± 0.8[b]	29.8 ± 1.0	29.9 ± 0.7
Brain, mg (% body weight)	453 ± 6 (1.36)	447 ± 22 (1.23)	448 ± 6 (1.18)[b]	476 ± 8 (1.63)	473 ± 11 (1.59)
Liver, mg (% body weight)	1571 ± 81 (4.59)	1879 ± 83[b] (5.12)[b]	2107 ± 74[b] (5.53)[b]	1537 ± 47 (5.22)	1439 ± 90 (4.77)
Spleen, mg (% body weight)	137 ± 12 (1.40)	147 ± 9 (0.40)	142 ± 6 (0.37)	159 ± 9 (0.54)	162 ± 10 (0.54)
Lungs, mg (% body weight)	204 ± 7 (0.61)	191 ± 8 (0.52)[b]	204 ± 8 (0.54)[b]	215 ± 11 (0.73)	190 ± 8 (0.64)
Thymus, mg (% body weight)	34 ± 2 (0.10)	45 ± 5 (0.12)	43 ± 4 (0.11)	54 ± 3 (0.18)	49 ± 3 (0.16)
Kidneys, mg (% body weight)	527 ± 18 (1.56)	557 ± 15 (1.52)	568 ± 15 (1.50)	410 ± 10 (10.40)	386 ± 16 (1.29)
Testes, mg (% body weight)	230 ± 9 (0.68)	241 ± 9 (0.66)	238 ± 12 (0.63)	—	—

[a] Values represent the mean ± SE.

[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

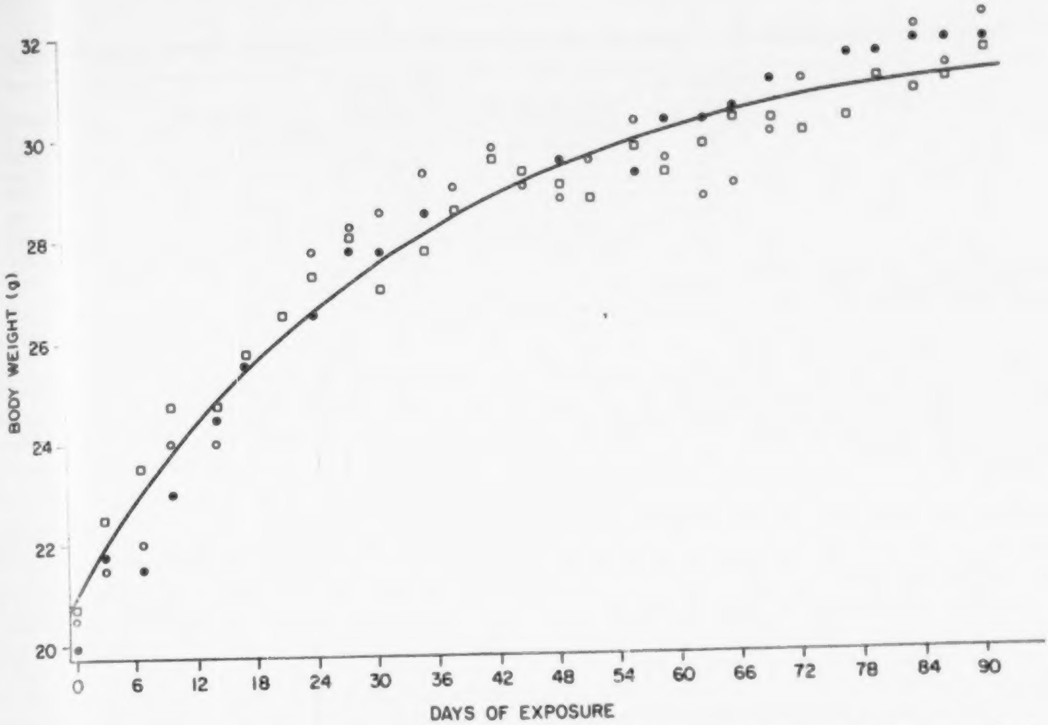

FIGURE 2. Growth chart of female CD-1 mice receiving chloral hydrate in the drinking water for 3 months. The mice received one of the following: (□) deionized water (48 mice) (0 chloral hydrate); (●) chloral hydrate (32 mice), 0.07 mg/ml (18 mg/kg/day); (○) chloral hydrate (32 mice), 0.7 mg/ml (173 mg/kg/day).

seen in the brain weights, which decreased proportionally with the increase in body weight.

Hematology

A summary of hematological findings is presented in Tables 8 and 9. Erythrocyte counts were 14% lower than control in female mice exposed to 0.7 mg/ml. This decrease was not confirmed with hemoglobin or hematocrit values, and was not evident in the males. Fibrinogen levels were elevated 9% above control in males exposed to 0.7 mg/ml, while a 14% elevation above control was seen in females exposed to 0.07 mg/ml. Slight increases were evi-

Table 8. Hematology of CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.[a]

	Males			Females		
Exposure Group	Deionized water (n = 21)	0.07 mg/ml (n = 14)	0.7 mg/ml (n = 16)	Vehicle (n = 22)	0.07 mg/ml (n = 13)	0.7 mg/ml (n = 16)
Hematocrit, %	45.5 ± 0.9	43.4 ± 0.9	43.4 ± 0.8	42.4 ± 0.4	42.6 ± 0.4	42.3 ± 0.8
Hemoglobin, g-%	12.7 ± 0.3	12.4 ± 0.3	11.9 ± 0.2	11.4 ± 0.2	12.0 ± 0.2	11.9 ± 0.3
Erythrocytes, 10^6/mm^3	11.0 ± 0.3	11.4 ± 0.3	11.0 ± 0.3	12.4 ± 0.4	11.9 ± 0.6	10.7 ± 0.2[b]
Leukocytes, 10^3/mm^3	9.09 ± 0.90	9.38 ± 0.87	10.23 ± 0.60	11.43 ± 0.61	12.7 ± 0.90	11.02 ± 0.49
Platelets, 1[illegible]/mm^3	3.98 ± 0.21	3.47 ± 0.22	4.20 ± 0.12	3.85 ± 0.19	3.94 ± 0.25	3.70 ± 0.18
Fibrinogen, mg-%	310 ± 10	321 ± 7	339 ± 9[b]	223 ± 5	265 ± 9[b]	244 ± 5
Prothrombin time, sec	9.7 ± 0.1	9.9 ± 0.4	9.7 ± 0.1	10.4 ± 0.2	10.4 ± 0.5	10.2 ± 0.3
APTT, sec	32.7 ± 1.8	34.2 ± 3.1	37.1 ± 1.8	33.7 ± 1.5	33.9 ± 2.4	38.1 ± 2.4

[a] Values represent the mean ± SE.
[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's T-test.

Table 9. Differential cell counts of CD-1 mice exposed to chloral hydrate in the drinking water for 3 mon[illegible]hs.[a]

Sex	Exposure group	Number of mice n	Lymphocytes	Polymorphonuclears	Monocytes	E[illegible]nophils
M	Deionized water	21	78.9 ± 2.6	18.8 ± 2.6	1.9 ± 0.5	0[illegible] ± 0.2
M	0.07 mg/ml	14	85.5 ± 1.7	10.6 ± 1.8[b]	2.4 ± 0.4	0[illegible] ± 0.3
M	0.7 mg/ml	16	83.0 ± 1.8	13.7 ± 1.7	1.5 ± 0.4	1[illegible] ± 0.3[b]
F	Deionized water	22	82.3 ± 1.9	14.0 ± 1.8	1.9 ± 0.3	1[illegible] ± 0.3
F	0.07 mg/ml	13	87.4 ± 1.2	9.5 ± 0.9	1.5 ± 0.3	1[illegible] ± 0.5
F	0.7 mg/ml	16	87.6 ± 1.6	9.4 ± 1.2	1.7 ± 0.4	1[illegible] ± 0.4

[a] Values represent the mean ± SE of the percent total of white cells.

[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

dent in the APTT times in both sexes; however, these increases were not significantly different from control. No consistent changes were observed in the differential counts.

Hepatic Microsomal Parameters

The status of the hepatic microsomal mixed functional oxidase parameters in mice exposed to chloral hydrate is summarized in Table 10. Microsomal protein was increased 10% above control in females exposed to 0.7 mg/ml, while no change was evident in males. Cytochrome b_5 levels increased 26% in males exposed to 0.07 mg/ml and 40% in males exposed to 0.7 mg/ml. In contrast, female mice exposed to 0.7 mg/ml had cytochrome b_5 levels 12% lower than vehicle control. Aminopyrine *N*-demethylase activity was increased in males exposed to 0.07 mg/ml (28%) and to 0.7 mg/ml (19%). No change in aminopyrine *N*-demethylase activity was evident in females. Aniline hydroxylase activity increased in both sexes. In males exposed to 0.[illegible]7 and 0.7 mg/ml, the increase in aniline hydroxylase activity was 24 and 30%, respectively; while a 2[illegible] increase was observed in females exposed to 0.7 mg/ml.

Serum and Liver Chemistries

Serum and liver chemistry values are presented in Table 11. In the sera of male mice receiving 0.7 mg/ml, calcium decreased 8%, sodium decreased 5%, phosphorous decreased 19%, LDH activity increased 48%, and SGOT levels increased 45% above controls. Blood urea nitrogen levels decreased 19% in males exposed to 0.07 and 32% in males exposed to 0.7 mg/ml. Female mice demonstrated a different pattern of changes in chemistry. In the sera of female mice exposed to 0.7 mg/ml, potassium increased 9%, glucose increased 16%, cholesterol increased 21%, and phosphorus increased 11% above vehicle control. No changes in serum enzyme

Table 10. Hepatic microsomal activities in CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.[a]

	Males			Females		
Exposure group	Deionized water (n = 4)	0.07 mg/ml (n = 8)	0.7 mg/ml (n = 8)	Deionized water (n = 8)	0.07 mg/ml (n = 4)	0.[illegible] mg/ml ([illegible] = 8)
Microsomal protein						
mg/g liver	23.0 ± 0.7	21.8 ± 0.8	21.0 ± 0.4	19.7 ± 0.4	21.0 ± 0.5	21.[illegible] ± 0.2[b]
mg/mouse	44.1 ± 2.6	41.1 ± 1.5	44.5 ± 2.7	31.9 ± 0.9	37.7 ± 3.8	39.[illegible] ± 1.7
Cytochrome P-450						
nmole/mg/protein	1.21 ± 0.13	1.32 ± 0.05	1.37 ± 0.05	1.23 ± 0.05	1.26 ± 0.06	1.1[illegible] ± 0.03
nmole/mouse	60.0 ± 2.1	54.1 ± 2.5	61.1 ± 5.0	39.3 ± 2.1	47.8 ± 6.4	44.[illegible] ± 2.2
Cytochrome b_5						
nmole/mg protein	0.402 ± 0.025	0.508 ± 0.022[b]	0.564 ± 0.017[b]	0.640 ± 0.021	0.662 ± 0.032	0.5[illegible] ± 0.024[b]
nmole/mouse	20.1 ± 6.9	20.2 ± 0.9	25.2 ± 1.9	20.4 ± 1.0	24.8 ± 2.0	22.[illegible] ± 0.6
Aminopyrine *N*-demethylase						
nmole/mg/min	9.47 ± 0.50	12.09 ± 0.27[b]	11.29 ± 0.32[b]	13.06 ± 0.46	13.09 ± 0.52	12.6[illegible] ± 0.31
nmole/mouse/min	418 ± 33	499 ± 34	500 ± 28	405 ± 19	490 ± 33	5[illegible] ± 30
Aniline hydroxylase						
nmole/mg/min	1.35 ± 0.05	1.68 ± 0.05[b]	1.75 ± 0.04[b]	1.70 ± 0.04	1.83 ± 0.17	2.0[illegible] ± 0.06[b]
nmole/mouse/min	59.6 ± 5.2	67.9 ± 3.1	77.1 ± 3.4	54.2 ± 1.3	68.3 ± 6.9	82.1 ± 1.6[b]

[a] Values represent the mean ± SE.

[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

Table 11. Clinal chemistry values of in CD-1 mice exposed to chleral hydrate in the drinking water for 3 months.[a]

	Males			Females		
Exposure group	Deionized water ($n = 22$)	0.07 mg/ml ($n = 14$)	0.7 mg/ml ($n = 16$)	Deionized water ($n = 23$)	0.07 mg/ml ($n = 14$)	0.7 mg/ml ($n = 16$)
Calcium, mg-%	11.74 ± 0.27	11.50 ± 0.39	10.76 ± 0.29[b]	11.19 ± 0.20	11.08 ± 0.24	11.62 ± 0.19
Sodium, meq/l.	151 ± 1	148 ± 1	143 ± 2[b]	144 ± 2	145 ± 2	151 ± 2
Chloride, meq/l.	107 ± 2	109 ± 1	111 ± 1	108 ± 1	114 ± 2[b]	108 ± 2
Potassium, meq/l.	6.24 ± 0.17	5.83 ± 0.22	5.97 ± 0.27	5.90 ± 0.10	6.07 ± 0.20	6.45 ± 0.24[b]
Protein, g-%	7.93 ± 0.19	9.08 ± 0.39	9.04 ± 0.71	8.14 ± 0.30	8.16 ± 0.40	8.26 ± 0.38
Glucose, mg-%	173 ± 5	166 ± 6	156 ± 10	120 ± 4	122 ± 6	139 ± 5[b]
Cholesterol, mg-%	87.8 ± 2.9	90.3 ± 3.4	88.2 ± 5.3	55.4 ± 1.8	61.0 ± 2.0	66.8 ± 2.3[b]
Phosphorus, mg-%	7.27 ± 0.14	6.99 ± 0.20	5.92 ± 0.25[b]	6.14 ± 0.11	6.10 ± 0.21	6.79 ± 0.18[b]
BUN, mg-%	30.9 ± 1.0	24.9 ± 1.0[b]	21.0 ± 1.1[b]	19.9 ± 0.5	19.4 ± 0.6	21.2 ± 1.1
LDH, IU/l.	741 ± 38	716 ± 40	1096 ± 171[b]	685 ± 33	704 ± 52	724 ± 36
SGPT, IU/l.	38.0 ± 1.6	36.0 ± 2.6	40.6 ± 2.7	28.9 ± 1.4	28.1 ± 1.5	32.6 ± 3.6
SGOT, IU/l.	94.1 ± 5.3	89.2 ± 6.6	136 ± 14[b]	89.4 ± 4.7	81.6 ± 6.2	93.8 ± 9.4
ALP, IU/l.[g]	48.2 ± 4.3	45.6 ± 2.0	42.6 ± 4.3	57.4 ± 2.4	53.3 ± 2.4	50.7 ± 3.4
GLU, μmole/g liver[h]	6.84 ± 0.52	7.21 ± 0.23	6.83 ± 0.27	6.01 ± 0.19	6.13 ± 0.21	5.34 ± 0.15[b]

[a] Values represent the mean ± SE.

[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

[c] BUN = blood urea nitrogen.

[d] LDH = lactic dehydrogenase.

[e] SGPT = Serum glutamic pyruvic transaminase.

[f] SGOT = Serum glutamic oxaloacetic transaminase.

[g] ALP = Alkaline phosphatase.

[h] Glutathione.

activities were evident in female mice exposed to chloral hydrate. Nonprotein sulfhydryl levels decreased 11% in females receiving 0.7 mg/ml, while no change was evident in male mice.

Discussion

The toxicity studies on chloral hydrate administered via the drinking water were undertaken because of its presence in human drinking water supplies (*1*). Acute, 14- and 90-day studies were performed on CD-1 random-bred mice. The acute lethal effects of chloral hydrate appear related to depression of the central nervous system. After oral administration of chloral hydrate, mice died within 24 hr without gaining consciousness; if they returned to consciousness, they survived. There were no sex differences in the LD_{50}'s of chloral hydrate. Boitsov (*12*) reported the LD_{50} in rats to be 285 mg/kg and the LD_{50} in mice to be 1100 mg/kg, which is very similar to the LD_{50} for mice obtained in our laboratories (see Table 1).

The 14-day study was performed in male mice with the chemical administered by stomach tube at doses 1/100 and 1/10 the LD_{50}. The exposure-related effects were an 18% increase in liver weight and a 27% decrease in spleen weight at the highest dose (144 mg/kg). These effects were not accompanied by any gross pathological changes.

In the 90-day drinking water studies, the highest time-weighted average dose the mice received per day was 173 mg/kg, based on fluid consumption and body weight. This is equivalent to a dose of about 14 mg/kg in humans when extrapolation is based on body surface area. For the average 60 kg person, this is less than 1 g/day. Thus, the doses administered in this study were high from an environmental standpoint, but were within the range of human therapeutic exposure. The currently recommended human therapeutic dose of chloral hydrate is 500-1000 mg, although doses of 2 g are not uncommon (*2*).

Chloral hydrate caused no deaths in the 90-day drinking water study, nor did it cause a decrease in weight gain. In the males a slight dose-dependent increase in body weight was observed.

In the male, the target organ appeared to be the liver. This is suggested by dose-related hepatomegaly, elevation in SGOT and LDH (but not in SGPT) activity, increased hepatic microsomal aminopyrine *N*-demthylase and aniline hydroxylase activity and increased ctyochrome b_5 content. However, there was no increase in cytochrome P-450 content.

Female mice did not exhibit the hepatomegaly observed in the males. However, at the highest dose, aniline hydroxylase activity was increased, while liver nonprotein sulfhydryl and cytochrome b_5 levels were decreased.

Neither sex showed exposure-related changes in hematological, coagulation, or urinalysis parameters. From the data presented the target organ for chloral hydrate appears to be the liver, particularly in the male mouse. The lowest adverse effect level was 0.07 mg chloral hydrate/ml of drinking water. The next report (5) will present evidence that the immune system may be more sensitive than the liver to the effects of chloral hydrate.

This work was supported by the Environmental Protection Agency (R806481010) and the National Institute of Environmental Health Sciences (IT32E507087).

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Environmental Health Perspectives
Vol. 44, pp. 147-151, 1982

Humoral and Cell-Mediated Immune Status in Mice Exposed to Chloral Hydrate

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Chloral hydrate has been found in our drinking water supplies at levels up to 5 μg/l. The purpose of this study was to evaluate the functional status of the immune system in random-bred CD-1 mice exposed to chloral hydrate for 14 and 90 days. Male mice, following 14 or 90 days of exposure to 1/10 and 1/100 the actual oral LD_{50}, exhibited no alterations in either humoral or cell-mediated immunity. However, female mice exposed for 90 days to chloral hydrate in the drinking water demonstrated a significant depression in humoral immune function. This depression was observed when spleen cells from exposed mice were evaluated for their ability to produce antibody against sheep erythrocytes. These females did not demonstrate any changes in cell-mediated immune status.

Introduction

This study describes the effects of subchronic exposure to chloral hydrate on the immune system. There has been renewed interest in chloral hydrate for, in addition to its use as a hypnotic in man, it is utilized industrially in the production of polyurethanes. Thus it enters the drinking water supply and has been found in six of ten water supplies examined by the EPA (*1*). Additionally, trichloroethylene, a common industrial solvent, is transformed into chloral hydrate during its metabolism (*2*); this provides two potential sources for absorption of chloral hydrate via the drinking water.

Little is known about the long-term effects of subchronic oral exposure to chloral hydrate. Therefore, this immunological investigation was conducted, as well as a general toxicological study, which was conducted concurrently and is described in a companion report (*3*). The parameters evaluated here are humoral immunity, determined by measuring plasma hemagglutination titers and the number of spleen antibody producing cells of mice sensitized to sheep erythrocytes; cell-mediated immunity, measured as delayed type hypersensitivity to sheep erythrocytes; lymphocyte responsiveness to T- and B-cell mitogens; bone marrow status, determined by cell number and DNA synthesis and the functional activity of the reticuloendothelial system, as measured by the vascular clearance rate and tissue uptake of ^{51}Cr sheep erythrocytes.

Methods

Exposure of Animals

Complete details concerning housing of the randomized CD-1 mice and their exposure to chloral hydrate are given in the preceding paper (*3*). The actual doses of chloral hydrate consumed as time-weighted averages from the two concentrations in the drinking water are also given in the preceding paper. In the 14-day gavage study, animals were sacrificed the day after the final dose. For the drinking water study, animals were maintained on chloral hydrate until sacrificed.

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Humoral Immunity

The primary IgM response to sheep erythrocytes was estimated by the hemolytic plaque assay of Jerne as modified by Cunningham and Szenberg (*4*). Mice were immunized by IP injection with 4 × 10^8 sheep erythrocytes (sRBC) 4 or 5 days prior to sacrifice. Spleen cell suspensions were prepared in RPMI 1640 culture medium and adjusted to a cell concentration of 5 × 10^5 cells/ml for assay of antibody forming cells (AFC).

As a complement to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Mice were injected IP with 10^9 sRBC 7 days prior to sacrifice and assay (day 7 for the 14-day study; day 83 of the 90-day study). An equal volume of a 0.5% suspension of sRBC was added to each of the dilutions in a microtiter dish. The antibody titers were expressed as $\log_2$ of the reciprocal of the first dilution where there was no visible agglutination.

Cell-Mediated Immunity

Cell-mediated immunity was evaluated by measuring the delayed type hypersensitivity response to sRBC. Sensitization was done 4 days before the end of the exposure period by injecting 10^8 sRBC in a volume of 20 µl into the left hind footpad.

Four days following sensitization (the last day of exposure), the mice were challenged in the same footpad with 4 × 10^8 sRBC in a volume of 40 µl. Seventeen hours following the challenge, the mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (Mallinckrodt) (80,000 cpm/0.1 ml). After 2 hr, the mice were sacrificed by cervical dislocation, and both hind feet were removed and placed in tubes for gamma counting. Paranjpe and Boone (*5*) have shown that ^{125}I-albumin extravasates into the extracellular space during the delayed type hypersensitivity response. The right footpad served as an unsensitized and unchallenged control for background infiltration of ^{125}I-albumin. A group of mice challenged with sRBC to determine nonspecific swelling served as unsensitized controls. Results are expressed as a stimulation index.

Lymphocyte Responsiveness to Mitogens

Lymphocyte responsiveness was assessed using the B-cell mitogen, LPS, which is lipopolysaccharide from *Salmonella typhosa* 0901 (Difco), and the T-cell mitogen, Con A, which is the plant lectin concanavalin A (Sigma). For mitogenicity assays, spleens were prepared as single-cell suspensions (5 × 10^6 ml) and 0.1 ml was added to three replicate wells of a microtiter dish containing mitogens. LPS was used in amounts of 5, 20, and 40 µg per well; Con A was used in amounts of 1, 5 and 10 µg per well. Plates were incubated at 37°C in 10% CO_2, 95% humidity, for 48 hr. The cells were then pulsed with an ^{125}I-iododeoxyuridine (IUdR; New England Nuclear)/fluorodeoxyuridine (FUdR; Sigma) solution (0.2 µCi IUdR in 1 µM FUdR). After an 18-hr incubation the cells were collected on filter disks, using a Titertek cell harvester, and counted in a Beckman gamma counter.

Bone Marrow Assay

To assess bone marrow DNA synthesis, bone marrow cells were collected from one femur, counted, and cells per femur determined. The cell count was adjusted to 3 × 10^6 cells/ml, and 200 µl of cells and 20 µl of ^{125}I-IUdR/FUdR solution were added to each microtiter well. At 60, 120 and 180 min, six replicate wells were harvested per animal, and the cpm's of IUdR incorporated into the acid-precipitable fraction were determined.

Functional Ability of the Reticuloendothelial System

Freshly drawn sRBC were radiolabeled with ^{51}sodium chromate (New England Nuclear). After chromation, the sRBC were washed with Alsever's solution until the supernatant fluid was virtually radioactivity free. Unlabeled sRBC (5 × 10^9 ml) were added to the labeled cells until the hematocrit was approximately 10%. The resulting cpm's were approximately 200,000/0.1 ml.

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 39°C. At 0 time, 0.1 ml of labeled particles/10 g body weight was injected intravenously. Blood samples (10 µl) were taken from the tip of the tail at 2, 4, 6, 8, 10 and 15 min. At the end of 60 min, the mice were sacrificed by decapitation, drained of blood, and organs were removed for radioassay. Blood clearance is expressed as the phagocytic index, which is determined by the slope of the clearance curve. Organ distribution is expressed as percent

Table 1. **Humoral immune response to sheep erythrocytes in CD-1 male mice exposed for 14 days to chloral hydrate.**[a,b]

Exposure group	Body weight, g	Speen weight		AFC/10^6 spleen cells	10^5 × AFC/spleen
		mg	% body weight		
Deionized water	29.8 ± 0.7	190 ± 13	0.64 ± 0.04	2286 ± 296	5.26 ± 0.7
14.4 mg/kg	31.4 ± 0.7	197 ± 14	0.62 ± 0.04	2324 ± 234	4.98 ± 0.6
144 mg/kg	31.2 ± 0.7	181 ± 10	0.58 ± 0.03	2210 ± 255	4.62 ± 0.6

[a] Mice were immunized on day 11 of gavage, and AFC's were determined 24 hr after day 14 of gavage.

[b] Values represent the mean ± SE derived from 11-12 mice/group.

organ uptake and cpm's/mg tissue (specific activity).

Statistical Evaluation

If a one-way analysis of the variance of the means showed treatment effects, a Dunnett's *T*-test was performed (*6*). Values which differ from vehicle control at $p < 0.05$ are noted with an asterisk in the tables. Each of the values in the tables is given as the mean ± standard error (SE) of the mean.

Results

Fourteen-Day Exposure to Chloral Hydrate

Chloral hydrate was administered to male CD-1 mice daily for 14 days by stomach tube at doses of 14.4 and 144 mg/kg (1/100 and 1/10 the LD_{50}). There were no significant changes ($p < 0.05$) in spleen weight, spleen antibody-forming cells, or delayed type hypersensitivity response to sRBC (Tables 1 and 2).

Ninety-Day Exposure to Chloral Hydrate

Male and female CD-1 mice received chloral hydrate in their drinking water for 90 days. Based on fluid consumption, chemical concentration, and body weight, time-weighted averages of chloral hydrate consumed were 16 and 160 mg/kg for males and 18 and 173 mg/kg for females (*3*).

Humoral and Cell-Mediated Immunity

The number of splenic antibody forming cells produced against sRBC in mice exposed to chloral hydrate for 90 days is summarized in Table 3. The peak day of response was day 4 after immunization for both sexes. On day 4, female mice produced 36 and 40% fewer AFC/spleen compared to control, at dose levels of 0.07 and 0.7 mg/ml, respectively. When expressed as AFC/10^6 spleen cells, the only significant decrease occurred at the high dose level of 0.7 mg/ml (173 mg/kg), where there was a 32% suppression. No changes were observed in male mice.

Serum hemagglutination titers were also measured. As seen in Table 3, no changes from control antibody titers were detected.

Delayed type hypersensitivity to sRBC was measured as an indicator of cell-mediated immunity (Table 2). Male mice exposed to the high dose level of 0.7 mg/ml demonstrated a 74% increase in footpad swelling compared to vehicle control, as measured by ^{125}I-albumin extravasation. On the other hand,

Table 2. **Cell mediated immune response to sheep erythrocytes in CD-1 mice exposed to chloral hydrate in the drinking water for 14 days or 3 months.**

Exposure time	Exposure group	Stimulation index	
		Males	Females
14 days[a]	Deionized Water	4.47 ± 0.50	–
	14.4 mg/kg	3.66 ± 0.48	–
	144 mg/kg	3.27 ± 0.49	–
3 months[b]	Deionized water	3.14 ± 0.45	4.57 ± 0.31
	0.07 mg/ml	2.77 ± 0.52	3.27 ± 0.35[c]
	0.7 mg/ml	5.48 ± 0.51	4.00 ± 0.42

[a] Mice were sensitized on day 10 of gavage, challenged on day 14, and measured for DTH on day 15. Values represent the mean ± SE derived from 12 mice/group.

[b] Values represent the mean ± SE derived from 17-20 mice in the deionized water group and 15-16 mice in the other groups.

[c] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

Table 3. Peak day humoral immune response to sheep eythrocytes and hemagglutination liters in CD-1 mice expo d to chloral hydrate in the drinking water for 3 months.

Sex	Exposure group	Humoral immune response[a]		Hemag utination titers ($_2$ titer)[b]
		10^5 × AFC/spleen	AFC/10^6 cells	
M	Deionized water	4.31 ± 0.52	2273 ± 132	9.4 ± 0.10
M	0.07 mg/ml	3.01 ± 0.55	2042 ± 125	9.7 ± 0.14
M	0.7 mg/ml	2.98 ± 0.28	1984 ± 167	9.7 ± 0.16
F	Deionized water	4.09 ± 0.30	2089 ± 82	9.3 ± 0.19
F	0.07 mg/ml	2.61 ± 0.39[c]	1695 ± 159	9.4 ± 0.31
F	0.7 mg/ml	2.47 ± 0.51[c]	1411 ± 199[c]	9.1 ± 0.19

[a] Values represent the mean ± SE derived from 12 mice in the deionized water group and 8 mice in the other grou
[b] Values represent the mean ± SE derived from 20-21 mice in the deionized water group and 13-16 mice in the oth groups.
[c] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

female mice exposed to the low dose level (0.07 mg/ml or 18 mg/kg) demonstrated a 28% inhibition in response.

Table 4 shows the responsiveness of spleen lymphocytes to the optimal concentrations of a T-cell mitogen (Con A) and a B cell mitogen (LPS). There were no exposure-related changes in lymphocyte response. Baseline response in the absence of mitogen is also shown in Table 4.

The functional activity of the reticuloendothelial system was not altered in male or female mice (data not shown). Even though there was a significant increase in kidney uptake of ^{51}Cr-labeled sRBC in female mice at the low dose, it was not considered biologically significant.

The number of bone marrow cells/femur was unchanged. There was a slight increase in bone marrow DNA synthesis in males as measured by incorporation of ^{125}I-IUdR (Table 5). This increase occurred at both exposure levels. It should be noted that DNA synthesis was higher in the control females, as compared to the males.

Table 4. Lymphocyte responsiveness in the absence and presence of mitogen using pleens from CD-1 mice exposed to chloral hydrate in the drinking water for 3 months.

Sex	Exposure group	No mitogen[a]	Con A (5 μg)[b]	LPS (20 μg)[b]
M	Deionized water	7419 ± 1294	258960 ± 6387	91291 ± 8977
M	0.07 mg/ml	7391 ± 731	253154 ± 8876	105541 ± 7851
M	0.7 mg/ml	8305 ± 1057	277594 ± 4954	88723 ± 8996
F	Deionized water	10697 ± 1439	260582 ± 9652	140246 ± 4903
F	0.07 mg/ml	7742 ± 1264	274527 ± 5593	138401 ± 4616
F	0.7 mg/ml	7569 ± 934	275839 ± 6699	129907 ± 6538

[a] Values represent the mean cpm's ± SE derived from 17-22 mice in the deionized water group and 13-16 mice in the o er groups.
[b] The optimum concentration of concanavalin A (Con A) and lipopolysaccharide (LPS) were employed. Values repres t the mean cpm's ± SE derived from 17-22 mice in the deionized water group and 13-16 mice in the other groups.

Table 5. Bone marrow synthesis in CD-1 mice exposed to chloral hydrate in the drinking water for 3 mon s.[a]

Exposure group	Males				Females			
	10^7 × cells/femur	60 min.	120 min.	180 min.	10^7 × cells/femur	60 min.	120 min.	180 min.
Deionized water	2.46 ± 0.17	2838 ± 246	5096 ± 385	6379 ± 429	1.99 ± 0.37	4475 ± 269	7084 ± 434	523 ± 360
0.07 mg/ml	2.36 ± 0.17	3692 ± 190[b]	6496 ± 409[b]	8142 ± 429	1.58 ± 0.11	4289 ± 259	7378 ± 418	164 ± 502
0.7 mg/ml	2.42 ± 0.20	4632 ± 283[b]	7526 ± 334[b]	8571 ± 403[b]	1.76 ± 0.17	4056 ± 294	6158 ± 274	269 ± 398

[a] Values represent the mean ± SE derived from 21 mice in the deionized water group and 13-16 mice in the other gr ups.
[b] Significantly different from control at $p < 0.05$ as determined by Dunnett's *T*-test.

Discussion

The previous report on chloral hydrate described the general toxicological effects of acute and subchronic exposure in random-bred CD-1 mice (*3*). Following exposure for 14 or 90 days, gross pathology, organ weights, clinical chemistries, hematology, urinalysis, and the effects on the hepatic microsomal enzymes were assessed. The most notable effects were in the livers of the male mice, where a dose-dependent hepatomegaly and an elevation in SGOT and LDH were seen. There was also increased hepatic microsomal aminopyrine *N*-demethylase and aniline hydroxylase activity, without an increase in P-450 content. In this report we describe the effects of chloral hydrate on the immune system when mice were exposed at the same time and under the same conditions as in the general toxicological studies.

The amount of chloral hydrate administered in the 90-day drinking water study was high in comparison to levels existent in the drinking water supplies surveyed by the EPA; that survey disclosed levels of chloral hydrate ranging from 2 to 5 µl/l. For the 90-day study, the highest dose administered to mice was 173 mg/kg. An equivalent human dose (based on body surface area) would be 14 mg/kg, or less than one gram. On a day-to-day basis, this could be considered a potentially harmful dose, especially in those individuals who are already at immunological risk.

The immune system in male mice showed no exposure-related depression or stimulation following 14 or 90 days of exposure. Humoral immune status, as assessed by measurement of the production of AFC, hemagglutination titers, and spleen cell response to LPS, was unchanged. Cell-mediated immunity, evaluated by a delayed type hypersensitivity response to sRBC, was enhanced in male mice exposed to the high concentration of chloral hydrate (160 mg/kg). The reason for this responsiveness may be related to the control stimulation index of 1.14 (Table 2), which was low in comparison to the mean for our accumulated historical control values for this strain of mouse. Spleen cell responsiveness to the T-cell mitogen concanavalin A was not altered, nor was there any effect on the functional activity of the reticuloendothelial system.

The immune status of female mice following 90 days of exposure differed from the males. The major difference was that humoral immunity, assessed by the number of AFC produced against sRBC, was depressed on the peak day of response (day 4). However, two other indicators of humoral immune status, hemagglutination titers and spleen cell response to the B-cell mitogen LPS, were not changed. The AFC response is the most sensitive indicator of humoral immunity because of the complex cellular cooperations required and because the number of cells that produce antibody can be quantitated. Although cell-mediated immunity was suppressed in mice exposed to the low dose of chloral hydrate, we do not believe this was compound related because the suppression was slight and it was not dose related. The effect on humoral immune status, concurrent with little alteration in the status of cell-mediated immunity, was consistent with results of other studies we have conducted upon water contaminants such as trichloroethylene and various chlorinated hydrocarbons (*7*). Why these compounds affect primarily the humoral immune functions is not clear.

From this study, it appears that the immune system, particularly the ability to produce IgM antibody to a T-dependent antigen in female mice, is the most sensitive indicator of the toxicity of chloral hydrate. The previous paper, which dealt with the toxicology of chloral hydrate, indicated that the liver is the most sensitive organ in male mice. The lowest concentration used, i.e., 0.07 mg/ml or about 16 mg/kg, was effective in eliciting both these responses.

This work was supported by the Environmental Protection Agency (R806481010) and the National Institute of Environmental Health Sciences (IT32E507087).

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Environmental Health Perspectives
Vol. 44, pp. 153-157, 1982

Alterations in Nerve and Muscle Compound Action Potentials After Acute Acrylamide Administration

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The early deficits of neurotoxicity induced by acrylamide were examined in rats by comparing nerve and muscle action potentials before and 24 hr after exposure to acrylamide (25, 50 or 100 mg/kg). No changes were seen in the nerve action potential amplitude or duration. The 25 mg/kg dose produced a more variable nerve conduction velocity. There was also a significant broadening of the muscle compound action potential. Neither of these effects were seen in the fasted controls. However, the lengthening of the relative refractory period of the muscle action potential was highly correlated with losses in body weight in the treatment groups and was identical to changes seen in control animals which were fasted for 24 hr. The slowed conduction of the muscle action potential may be a precursor of the nerve terminal damage which results from chronic exposure. Changes in the muscle refractory period, on the other hand, appear to be secondary to the loss in body weight which accompanies acrylamide administration.

Introduction

Chronic exposure to acrylamide results in progressive signs of neurotoxicity which have been described in both morphologic (*1*) and behavioral terms (*2*). The effects on various components of peripheral nerves have been the most extensively studied aspects of acrylamide toxicity. There is a selective loss of the distal portions of both motor (*3*) and sensory nerves (*4*, *5*), with the large diameter fibers affected before smaller axons (*6*).

Clinically, acrylamide toxicity is characterized by paresthesia (*1*) and muscle weakness (*2*). Although these effects are related to the neuropathy, it is important to note that behavioral deficits have been reported prior to the appearance of histologic nerve damage. Some behavioral changes occur as a result of an effect on membrane excitability. However, the electrophysiologic characteristics of peripheral nerve and skeletal muscle are attenuated by acrylamide only after long periods of chronic dosing, an effect which correlates more closely with the destruction of nerve axons (*7–9*) than with behavioral deficits.

Unfortunately, the effects of acrylamide on electrophysiologic parameters have usually been examined using indirect, noninvasive techniques. Inaccuracy in these measurements probably masked any subtle effects of the drug on axonal and muscle excitability. With more accurate techniques it might be possible to demonstrate that the muscle weakness and paresthesia following acrylamide administration are linked to a loss of electrophysiologic responsiveness in the nerve and/or muscle, and that both effects precede the histologic damage which occurs only after long-term administration of acrylamide.

The purpose of this study was to examine whether changes could be detected in the electrophysiologic activity of peripheral nerve and muscle using direct measurements after relatively short-term exposure to acrylamide.

Methods

To reduce the variability in measurements, each animal in these experiments served as its own

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control. The control parameters of nerve and muscle activity were recorded from the left hindlimb. One week later the animal was dosed with acrylamide. Twenty-four hours after acrylamide administration, another set of parameters was recorded from the contralateral hindlimb.

Male Sprague-Dawley rats (190-240) were anesthetized with 50 mg/kg pentobarbital, adminstered intraperitoneally. The left hindlimb was dissected to expose the sciatic nerve and triceps surae muscles. Pairs of platinum-iridium stimulating and recording electrodes were placed on the sciatic nerve for obtaining control measurements from the nerve. The muscle electrophysiologic activity was also recorded differentially. Following the data collection the hindlimb was sutured and the animal was allowed to recover from anesthesia. Care was taken not to sever or damage any of the major hindlimb innervation during these procedures and cursory observation of the animal after recovery from anesthesia revealed no detectable deficit in locomotion as a result of these procedures. One week was allowed for the animal to recover from the effects of the surgery.

Groups of five animals from which control measurements had been taken were then dosed with either 25, 50 or 100 mg/kg of acrylamide or 1 ml saline intraperitoneally. At 24 hr after administration, each animal was again anesthetized with 50 mg/kg pentobarbital and the right hindlimb was dissected. The right sciatic nerve and triceps surae muscles were recorded in a similar manner to the left.

Following this set of electrophysiologic recordings, the sciatic nerves from both limbs were fixed *in situ* in 10% formalin for histologic examination. After staining with hemotoxylin-eosin the nerves were cut in both longitudinal and cross section and mounted. The coded slides were evaluated by an unbiased morphologist for evidence of structural abnormalities.

Based on pilot studies, the acrylamide treated animals were expected to lose a significant amount of weight. In order to account for effects which might be caused by a nonspecific and acute weight loss, control animals were fasted for 24 hr before recording from the contralateral hindlimb.

The procedure for recording the electrophysiologic parameters before and after acrylamide has been described previously (*10*). Supramaximal rectangular pulses (0.02 msec duration) were applied to the sciatic nerve at 15- sec intervals. The nerve and muscle action potentials were recorded from 100 consecutive stimuli. Each response was converted to its digital equivalent, analyzed and stored by our microprocessor system. The muscle action potential latency was measured as the time in milliseconds from the stimulus artifact to the onset of muscle depolarization. The nerve conduction velocity was calculated from the length of nerve between the stimulating and recording electrodes and the conduction time. For both the nerve and muscle action potentials, duration was measured from onset of depolarization to return to the baseline; peak amplitude was calculated in millivolts.

Twin pulse stimuli were used to determine the refractory period of the muscle action potential. As the stimulus interval was varied between 3.5 and 20 msec, the two muscle action potentials were recorded and analyzed. The relative refractory period was taken as an exponential function (*11*) of the second action potential relative to the first. From these functions, the time at which the second potential reached 75% of its maximal amplitude was calculated and was taken as a standardized measure of refractoriness for comparison between treated and control groups.

Changes in each parameter of the action potentials between the left and right hindlimb were analyzed as a function of dose by analysis of covariance. In addition, the refractory period measurements were analyzed by the sign test. Changes which differed at $p < 0.05$ were judged to be statistically significant.

Results

The effects of acrylamide on the nerve and muscle action potentials are summarized in Table 1. The only significant change in nerve activity was an increased variability in conduction velocity after 25 mg/kg. The other characteristics of the wave form at this dose and all characteristics at the 50 and 100 mg/kg does were unaffected by acrylamide.

Alterations in the muscle action potential after acrylamide administration can be seen qualitatively as a change in the shape of the wave form. One such example is shown in Figure 1. Although there was no significant change in the conduction velocity which was calculated from the onset of the action potential depolarization, there was a marked broadening of the wave form. This was confirmed statistically as an increase in the duration of the muscle action potential as indicated in Table 1 and was seen after all three doses of acrylamide.

Acrylamide also induced a prolongation and increased variability of the relative refractory period of the muscle action potential. The stimulus interval required for 75% recovery of the action potential in each treatment group is compared in Table 2. With the contralateral untreated side being used as

Table 1. Effects of acrylamide and fasting on nerve and muscle electrophysiologic activity.

	Control, unfasted	Control, fasted	Acrylamide 25 mg/kg	Acrylamide 50 mg/kg	Acrylamide 100 mg/kg
Nerve action potential					
Conduction velocity, m/sec	31.7 ± 1.4	35.8 ± 3.2	79.0 ± 24.9[a]	38.0 ± 3.2	34.0 ± 3.5
Duration, msec	1.05 ± 0.09	0.83 ± 0.03	1.02 ± 0.13	0.94 ± 0.29	0.85 ± 0.35
Amplitude, mV	0.49 ± 0.07	0.99 ± 3.11	0.42 ± 0.15	0.72 ± 0.10	0.92 ± 0.10
Muscle action potential					
Latency, msec	1.87 ± 0.06	2.06 ± 0.21	1.81 ± 0.11	1.79 ± 0.22	1.76 ± 0.23
Duration, msec	1.96 ± 0.13	1.98 ± 0.11	2.58 ± 0.33[b]	2.42 ± 0.25[b]	2.48 ± 0.26[b]
Amplitude, mV	0.58 ± 0.05	0.68 ± 0.20	0.51 ± 0.09	0.58 ± 0.15	0.81 ± 9.20

[a] $p < 0.0$, compared with unfasted control.
[b] $p < 0.0$, compared with unfasted control.

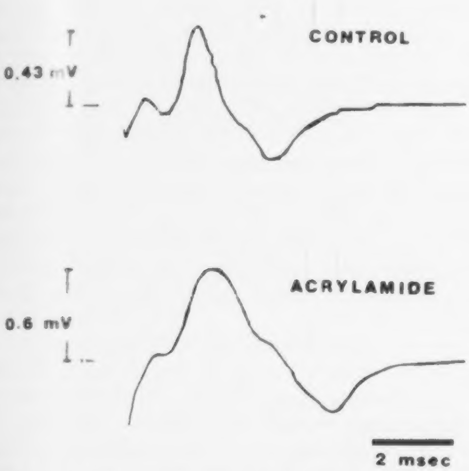

FIGURE 1. Effect of acrylamide on the muscle compound action potential. Each panel shows the computer average of 100 consecutive action potentials: (top) control response of one animal; (bottom) response from the contralateral limb of the same animal 24 hr after exposure to 50 mg/kg acrylamide.

the control, acrylamide (100 mg/kg) significantly increased the refractory period from 5.25 to 8.85 msec. Interestingly, control rats which were fasted for 24 hr also showed a marked prolongation in the relative refractory period which was indistinguishable from the rats receiving 100 mg/kg acrylamide. Concurrently with this prolongation, the refractory period was more variable among the treated animals than in the same animals prior to treatment. Although the increased variability only reached statistical significance for the fasted animals and the 100 mg/kg acrylamide-treated animals, the standard errors are clearly larger in all treated groups, compared to the unfasted controls.

As we had seen previously in pilot studies, acrylamide treatment resulted in a loss of body weight. A significant loss of approximately 19 grams was seen in the animals receiving 100 mg/kg acrylamide and in the control rats which were fasted for 24 hr. This is shown in Figure 2. The loss in body weight for all groups was correlated statistically with the prolongation in the muscle action potential relative refractory period (correlation coefficient $r = 0.96$).

There are several incidental observations which should also be noted. The acrylamide treated animals, especially those receiving 50 or 100 mg/kg, showed signs of behavioral toxicity after 24 hr of exposure. Although these were not quantitated, the animals appeared to be hyperreflexic, especially to nonspecific somatosensory stimulation. They also resisted being handled. Resting tremor was noted in several animals receiving 100 mg/kg but locomo-

Table 2. Effect of acrylamide and fasting on the relative refractory period of the muscle action potential.

	75% Refractory period, msec (mean ± SEM)	% of unfasted control	Sign test[a]
Control, unfasted	5.25 ± 0.51	100	
Acrylamide			
25 mg/kg	7.4 ± 2.70	114	NS
50 mg/kg	6.4 ± 1.20	120	NS
100 mg/kg	8.85 ± 2.79	184	$\alpha < 0.01$
Control, fasted	8.82 ± 2.87	200	$\alpha < 0.01$

[a] Compared with unfasted control.

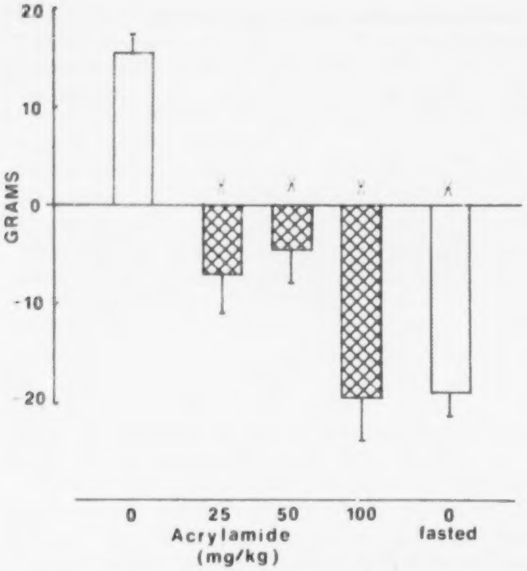

FIGURE 2. Effect of acrylamide on body weight. Each bar is the mean ± SEM of the weight lost or gained in each group during the 24 hr treatment period. The cross-hatched bars show the acrylamide-treated groups and the stippled bars show the control groups. The control group on the right was fasted for 24 hr. Asterisks (*) indicate $p < 0.05$ when compared to the untreated control.

tion was not markedly altered in any of the treated animals.

No sign of peripheral neuropathy was noted in the light microscope slides of either hindlimb nerve at any of the three acrylamide doses. In some cases, however, there was evidence of increased vascularization and an inflammatory response on the left side, which was the first operated side. The sciatic nerves from the right hindlimb, which were fixed immediately following the electrophysiologic recording, were normal.

Discussion

These results show that alterations in nerve and muscle function can be detected after only 24 hr exposure to acrylamide. Although an accurate correlation was not attempted between the electrophysiologic effects and changes in the animals' behavior, it was noted that the animals receiving 50 or 100 mg/kg also showed behavioral signs of acrylamide intoxication (tremor, hyperreflexia, etc.).

It is important to note that the changes in the muscle action potential were more pronounced than in the nerve and suggests that the initial effects of
acrylamide are on muscle function
nerve. This would be consistent with t
tions of Tilson and Cabe (*2*), who have re
hindlimb muscular weakness is the ear
acrylamide intoxication. It is interest
that Leswing and Ribelin (*8*) reported
prolongation of conduction distally thr
terminals and muscles of cats and mo
acrylamide administration. Since the n
lar junction is affected only after longe
exposure to acrylamide (*12*), our observ
with others suggest that the "dying b
axons actually begins with a change in
loss then proceeds to the motor nerv
which are affected before motor axons

Only one change was noted in the sc
an increased variability in the conduct
after 25 mg/kg. This effect is difficult
since larger doses had no effect. It may
er, that the low dose produced a tran
atory response which is unrelated to
quent peripheral neuropathy. Several o
toxic agents have been shown to be biph
effects (*13*), producing an initial hyper
and then a more pronounced and prolon
sant effect. Only the latter, incidently
correlated with structural damage to t
system (*13*).

It is important to note that both these c
nerve and muscle—were demonstrated
24 hr exposure to acrylamide. The func
trophysiologic deficit appeared to cor
our uncontrolled observations of behavio
in the high dosed animals. However,
tional deficits occurred prior to any
pathology in the nerve. Only one st
behavioral techniques (*2*), has shown
function with an earlier onset (12 hr aft
to 200 mg/kg). However, no mention w
this case of weight loss, which would be
be great, and one can question the con
generalized weakness to the result.

In our study, the above-mentioned
significant because they did not occur
animals which had been fasted to lose
weight over the same time period. T
trasted by the effects on the muscle
period in which there was an obvious
with weight loss. This increased refra
the muscle may contribute to the gros
observed in animals after short-term
does not preclude other factors (e.g.
from contributing to the motor dysfu
after longer term exposure. In any cas
important effect is the slowed muscle
caused by acrylamide.

Others have achieved a separation of muscle dysfuncti n and weight loss (2), but only after very low dose and at least one week of chronic exposure. In v ew of this, the electrophysiologic changes after 24 r are all the more striking. One can speculate hat these early, subtle changes in the muscle, a d perhaps the nerve, are the first indication tha a progressive distal-to-proximal neuromuscular ysfunction has begun, and if allowed to continue ill result in structural damage.

The auth wishes to thank Dr. J. J. Bernstein for assessing the nerve orphology and Mr. John Parker and Mr. Victor Church for ssisting with the technical portions of this work. This study as supported by NIH grant ESO1951.

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Environmental Health Perspectives
Vol. 44, pp. 159-164, 1982

Children's Blood Lead Levels in New Haven: A Population-Based Demographic Profile

by Ruth Fitch Quah,* Alice D. Stark,† J. Wister Meigs‡ and Edward R. DeLouise**

This report presents the geometric mean blood lead levels of an 80% cross-sectional sample of children aged 1-72 months in New Haven, Connecticut. Blood lead levels were related to age, sex and race. It was found that age and race were independently important sources of variation in blood lead levels. Sex of children in this age group was not related to differences in blood lead levels. The highest geometric mean blood lead levels occurred in children between 25 and 36 months of age. Black children had higher levels than white or Hispanic children.

Introduction

In the extensive literature on childhood lead poisoning there is little descriptive information about blood lead levels among urban children divided by age, sex and race. Numerous studies have focused on children at highest risk or children with undue lead absorption. Indeed, Chisolm has stated that there are, as yet, no studies designed to measure incidence on the basis of sound epidemiological principles (*1*).

Several estimates of blood lead in U.S. children have been published. They vary widely. Gilsinn (*2*) derived national estimates by using data from the 1970 census for housing built before 1950 and blood lead data from eastern and midwestern large urban areas. She estimated a nationwide rate of 28.9% of children with blood lead concentrations ≥ 40 μg/dl, 12.7% ≥ 50 μg/dl, 6% ≥ 60 μg/dl, and 2.7% ≥ 70 μg/dl. In contrast, a recent Center for Disease Control report (*3*) stated that since 1972 lead poisoning prevention programs located in all sections of the country have reported the screening of 3,350,000 children, ages 1-5; 221,000 (6.6%) have been identified as having blood lead levels ≥ 30 μg/dl.

A number of factors have been identified as influencing the likelihood of an increased body burden of lead. These include age, race, socioeconomic status, housing and season. Sex does not seem to be an important factor (*4*, *5*). Children from one through six are at risk for childhood lead poisoning, with peak incidence occurring between one and three years (*6*).

It is not clear whether race acts independently or merely in association with other factors. Many studies have looked only at high risk populations where racial distribution is highly imbalanced. Jacobnizer (*7*) reported a disproportionately high incidence of elevated blood lead levels in the Puerto Rican and nonwhite groups in a high risk neighborhood screening program. Such neighborhoods are usually characterized by slums and dilapidated housing and children living there are generally at

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greater risk for increased lead absorption than are those in better quality neighborhoods (*7, 8*). Incidence of cases of acute childhood lead poisoning peaks in the summer months (May-October) for a mixture of reasons, including sunshine, dietary calcium, vitamin D, dehydration and availability of lead from outdoor paint, soil and fallout from the air, as well as other factors not yet understood (*3, 8-10*).

Methods

The Health Department of the City of New Haven carried out a screening program from July 1974 to February 1977. All children residing within the city who were 1 through 72 months old were eligible for screening. A variety of publicity approaches were utilized to inform parents about the screening program and to urge participation. The Health Department provided the screening test free of charge at a number of locations throughout the city, including in a mobile van and in the childs' own home, if desired. Parents who chose not to use these services were encouraged to have their children tested by their own physicians. All blood lead tests in New Haven, regardless of who performs them, are required to be reported to the Health Department.

The purpose of the program was explained to the parent(s) or guardian of each child to be included. Written consent was obtained from parent or guardian before each test was performed. Detailed information was obtained on a cross section of the population of interest (*11*). This study group was composed of 7912 children in the age group 1 through 72 months of age at the time of test and represented about 80% of the entire 1-72 month old resident pediatric population during this time period. Of the remaining 20%, approximately 1% comprised children who were screened but not included in this report because their racial designation was other than black, white or Hispanic. The other 19% were not proportionally distributed and tended to be of higher socioeconomic status and to be white. This resulted from the less intense effort to screen children thought not to be at risk for lead poisoning. For each child in the sample a blood lead measurement was obtained.

All sample collections by the New Haven Health Department were done by either of two individuals, a certified laboratory technician or a registered nurse who had been trained by the technician. The sampling technique was standardized as follows. The child's middle finger was thoroughly cleansed with soap solution, rinsed and dried. The finger was then sprayed with collodion, which was allowed to dry. A disposable lancet was used to puncture the finger from which approximately ten drops of blood were allowed to drip onto the filter paper which was then inserted into a plastic envelope. The envelope was sealed and refrigerated for a maximum of 24 hr before being mailed to the State Health Department Laboratory. Collections by other medical care providers were done by nurses, physicians or trained technicians. Samples were collected in individual homes as well as clinics, hospitals and private care facilities. With the exception of Yale-New Haven Hospital, all medical care providers sent their blood samples to the Connecticut State Health Department Laboratory. The State used the Dithizone method (*12*) of analysis for macro (5-10 ml) samples and atomic absorption spectrophotometry with Delves' cup attachment (*13*) for micro samples. The State Laboratory has been cooperating with the Childhood Lead Poisoning Control Laboratory Monitoring Program of the Center for Disease Control (CDC). Based on the results of this monitoring mechanism, performance of the State Laboratory has been evaluated as acceptable or better. Yale-New Haven Hospital performed only macro tests and analyzed them using the atomic absorption method (*14*). The hospital laboratory meets state established quality control standards and also participates in the CDC proficiency blood lead testing program.

The reliance placed on blood lead measurement as the indicator of lead absorption in the New Haven study population made it necessary to assess the accuracy of the screening results, and particularly to assure that contamination of micro blood tests had not occurred. When micro test results were compared with macro test results (which were assumed to be accurate), after appropriate matching for age, race and date of sampling, there was no evidence to indicate any substantial inaccuracies of micro results or inconsistencies between results obtained by the two laboratories (*15*). For 410 children the micro test was followed within 60 days by a macro test. The mean difference in results was -7.1 μg/dl. This difference is not significant ($p > 0.05$) and could well have come about from an actual decrease or the error level inherent in the test.

The blood lead measurement is generally accepted as the best indicator of the external dose of lead, (*16*) where the external dose is the concentration of lead in environmental sources. Blood lead measurements are highly useful in the study of groups because the average blood lead level in a group reflects well the levels of current exposure and absorption of lead for the group as a whole (*17*).

Several authors have suggested that the distribution of blood lead levels for any relatively homogenous po ulation closely follows a lognormal distribution (1 20). A variable is said to have a lognormal distribut n if the logarithm of the variable is normally distributed. The skewed nature of the lognorm distribution makes the median (50th percentil) a more meaningful estimate of central tendenc han the arithmetic mean. For the normal distribut n, the best estimate of the median is $\bar{x}$, the sim arithmetic mean. For the lognormal distribut n, the best estimate of the median (*21*, *22*) is th geometric mean

$$GM = \text{antilog}\left[\sum_{i=1}^{n} (\log x_i/n)\right]$$

For this eason, the blood lead data presented in this repo t are geometric means.

Results

Geometric mean blood lead levels were stratified by the following factors: age, sex by age, race by age. From the 7912 intial tests available, analysis was based on tests on 7520 children in the study population for whom no measurements of environmental lead exposure had been obtained. Fifteen children were excluded because of missing information. Data for the remaining 377 children for whom environmental lead levels were determined have been reported upon separately (*23*). Exclusion of these 377 children had no effect on the results presented in this report.

Age

The initial analysis was a breakdown of geometric mean blood lead level of 7520 children by age in

Table 1. Geometric mean blood lead levels by age of children in New Haven, Conn., July 1, 1974–Feb. 28, 1977.

Age, months	Number of children	Blood lead level, µg/dl GM[a]	GSD[b]	Range
1–12	663	25.5	1.4	8.0–97.1
13–24	1557	28.3	1.4	0.0–80.9
25–36	1198	28.7	1.4	0.0–75.0
37–48	1284	27.8	1.4	7.0–73.0
49–60	1365	27.4	1.4	3.0–74.0
61–72	1453	26.1	1.4	0.0–69.0
Total	7520	27.4	1.4	0.0–97.1

[a] GM = geometric mean blood lead level.

[b] $GSD = \text{antilog}\left\{\sum_{i=1}^{n} [\log(x_i) - \log(GM)]^2/(n-1)\right\}^{1/2}$

Table 2. Geometric mean blood lead levels in children aged 1-12 months in New Haven, Conn., July 1, 1974–Feb. 28, 1977.

Age, months	Number of children	Blood level, µg/dl GM[a]	GSD[b]	Range
1	22	26.1	1.4	14.0–45.0
2	6	25.8	1.2	23.0–32.0
3	3	25.1	1.2	22.0–31.0
4	6	28.6	1.7	17.0–72.0
5	14	25.0	1.3	16.0–43.0
6	13	26.5	1.4	14.0–39.0
7	29	23.2	1.3	12.0–36.0
8	51	25.8	1.4	10.0–50.00
9	110	24.5	1.4	9.0–40.0
10	83	24.6	1.4	8.0–45.0
11	123	25.2	1.4	9.0–97.1
12	218	26.8	1.4	10.0–63.0
ll ages	678	25.5	1.4	8.0–97.1

[a] GM = ometric mean blood lead level.

[b] $GSD = \text{ntilog}\left\{\sum_{i=1}^{n} [\log(x_i) - \log(GM)]^2/(n-1)\right\}^{1/2}$

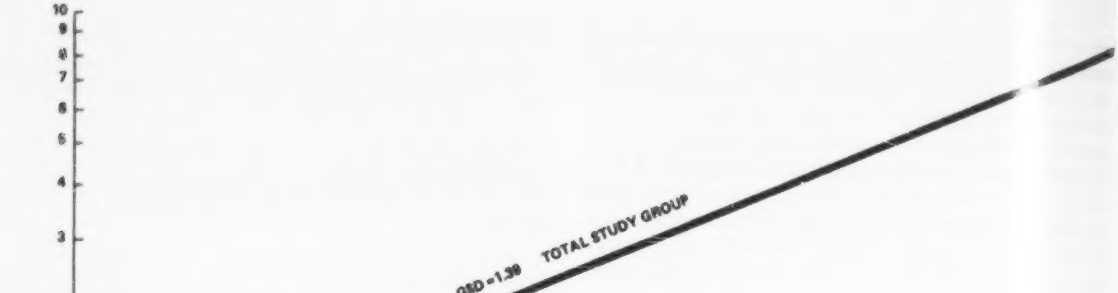

FIGURE 1. Estimated cumulative distribution of blood lead levels for the total sample having a geometric mean blood lea level of 27.5 μg/dl and geometric standard deviation of 1.39.

Table 3. Geometric mean blood lead levels by race and age of children in New Haven, Conn., July 1, 1974–Feb 8, 1977.[a]

Age, months	Black			Hispanic			White			Difference at 0.0	ignificant evel
	Blood lead, μg/dl			Blood lead, μg/dl			Blood lead, μg/dl				
	GM	GSD	N	GM	GSD	N	GM	GSD	N		
1–12	27.2	1.3	275	24.4	1.3	108	22.7	1.4	124	None	
13–24	31.5	1.4	660	28.6	1.4	228	24.4	1.4	319	Black –	hite
25–36	30.9	1.4	531	28.5	1.4	186	25.3	1.5	264	None	
37–48	29.2	1.4	628	29.6	1.4	169	24.3	1.4	298	None	
49–60	29.2	1.3	704	28.2	1.3	189	23.6	1.4	361	Black –	hite
61–72	28.6	1.3	648	27.3	1.4	213	22.9	1.3	541	Black –	hite
Total	29.6	1.4	3446	27.4	1.4	1093	23.8	1.4	1907	Black –	hite

[a] GM = geometric mean blood lead level.

[b] $\text{GSD} = \text{antilog} \left\{ \sum_{i=1}^{n} [\log (x_i) - \log (\text{GM})]^2 / (n - 1) \right\}^{1/2}$

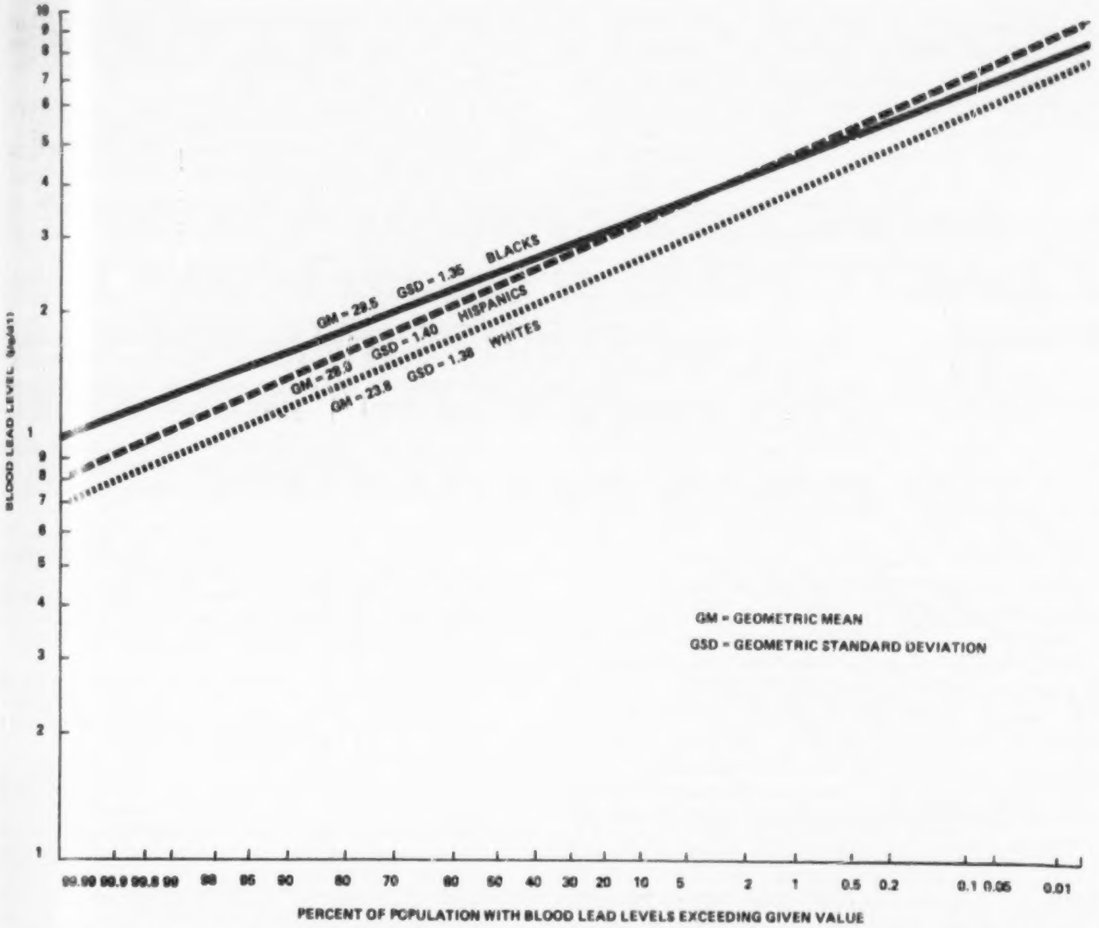

FIGURE 2. Estimated cumulative distribution of blood lead levels for blacks having a geometric mean of 29.5 µg/dl and geometric standard deviation of 1.35, for whites having a geometric mean of 23.8 µg/dl and geometric standard deviation of 1.38, for Hispanics having a geometric mean of 28.0 µg/dl and geometric standard deviation of 1.40.

months, at the time of test, as follows: 1-12; 13-24; 25-36; 37-48; 49-60; 61-72. It can be seen in Table 1 that the highest mean blood lead levels were between ages of about 25-36 months. The mean of the entire sample was 27.4 µg/dl with a standard deviation of 1.39. The range of blood lead levels by age category is considerable. Table 2 shows the distribution of geometric mean blood lead levels for the children in the 1-12 month age category by single month of age. Of the 678 children in the 1-12 month category, 614 were between 7-12 months old. The mean level for the 1-6 month old children was 26.1 µg/dl compared to a mean of 25.5 µg/dl for the 7-12 month old children, an insignificant difference. Figure 1 shows that 3.5% of the sample population had blood lead levels ≥ 50 µg/dl and 39% had levels ≥ 30 µg/dl.

Sex by Age

Sex was recorded in 7462 of 7520 cases. When mean blood lead levels were compared by age and sex, the levels of the two sexes did not differ significantly. Therefore, results for both sexes were combined in subsequent analyses.

Race by Age

Genetic characteristics were not defined in this study, but family self-definition was used to assign each child to a racial/ethnic/cultural category. Although there were a small number of people who did not classify themselves as belonging to one of the three major racial groups in New Haven (black, white, Hispanic), the "other" category was too small to be included in analysis. Race data were missing in about 14% of subjects. Table 3 gives the results of geometric mean blood lead levels by race and age. For all ages the lead levels of whites were substantially lower than those of blacks and Hispanics. Until age 37-48 months, levels for Hispanics were lower than for blacks, but starting with that age, the levels of these two groups were similar. Between the ages of 18-42 months, the geometric mean blood lead level for black children in New Haven was greater than 30 μg/dl.

Comment

A geometric mean (i.e., a median) blood level of 27.4 μg Pb/dl in an 80% sample of the entire population of New Haven children aged 1-72 months shows that a good deal of lead was available to them. Much of the lead found in these children's blood can be presumed to have come from the external physical environment rather than from food or beverages. This inference is supported by the peak values in children between about 18 and 42 months of age, when normal mouthing behavior would be expected to maximize the oral intake of nonfood materials. Contamination of these materials with even small amounts of lead would produce the results observed. The source of the high levels of lead found in the blood of very young infants is less clear. Prenatal exposure cannot be ruled out. The implications of these high blood lead levels in terms of effect on the vulnerable developing nervous system are cause for concern.

The substantial differences in mean blood lead levels among different groups classified by racial category indicate the importance of this variable. Figure 2 shows the estimated cumulative distributions of blood lead levels for black, white and Hispanic children. It can be seen that 50% of the black children, 43% of the Hispanic children and 23% of white children had blood lead levels ≥ 30 μg Pb/dl. This leaves no room for complacency. Similar population surveys in other areas are needed to place the New Haven results in a broader prospective. A random sample of adults should be included. The design and implementation of prevention and treatment strategies for childhood lead poisoning will require ongoing surveillance of populations whose demographic characteristics are clearly defined.

This work was supported in part by Grant #01-H-000278-02-0, United States Department of Health and Human Services, Public Health Service and Grant # 2549-RG, United States Department of Housing and Urban Development.

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Environmental Health Perspectives
Vol. 44, pp. 165-174, 1982

A Study of Acute Respiratory Disease in Families Exposed to Different Levels of Air Pollution in the Great Salt Lake Basin, Utah, 1971-1972 and 1972-1973

by Gory J. Love,* Shu-Ping Lan* and Carl M. Shy*

The reported incidence of acute respiratory illness in families exposed to different concentrations of air pollution was studied during two consecutive school years. The purpose of the study was to determine the effect of increased exposure to sulfur dioxide and suspended particulate matter. In each of four study communities, the mothers of approximately 250 white families were contacted biweekly to obtain information regarding the occurrence of respiratory symptoms in each family member. Annual mean ambient sulfur dioxide concentrations in one community for the three years included in the study (1971-1973) were well above the current air quality standard of 80 $\mu g/m^3$, while in the other three communities the annual sulfur dioxide concentrations were much lower (usually less than 40 $\mu g/m^3$). Suspended particulate matter concentrations in the high sulfur dioxide community were close to the limit designated by the annual standard (75 $\mu g/m^3$) but actual exposures in the four communities probably were not excessive. Regression analyses of the data collected showed inconsistent associations between illness rates and educational attainment of the head of household, crowding in the home, bronchitis in parents or smoking by parents. However, once the effects of these factors were removed the adjusted rates showed little association with community of residence. It was concluded that the higher concentrations of sulfur dioxide in the Utah atmosphere could not be the cause of increases in acute respiratory illness in the exposed populations.

Introduction

The occurrence of acute respiratory illness in populations exposed to differing concentrations of air pollution was studied in Utah during the 1971-72 and 1972-73 school years. The studies conducted by the Health Effects Research Laboratory of the U.S. Environmental Protection Agency were undertaken in an effort to provide additional information relevant to effects of exposure to atmospheric sulfur dioxide (SO_2) and suspended particulate matter (TSP).

A number of studies related to this subject have been reported from the United States and from England (*1-14*). These have been consistent in their findings of higher incidences of lower respiratory illness in populations exposed to relatively high concentrations of ambient air pollution. Less consistently, upper respiratory illness also has been associated with such exposure. At least some of these studies also demonstrate the effect of socioeconomic factors on respiratory illness rates (*12*, *13*).

A retrospective study of lower respiratory illness in the same study areas in Utah (*15*) found that children who had resided in the high pollution area for three years or more had more illness than did their counterparts in the less polluted communities. However, no association between pollution exposure and illness was found for children with residence times of less than three years. The prospective studies reported here were designed to investigate whether higher rates for acute respiratory disease (ARD) persisted in the high pollution area or if the lack of association in shorter term residents reported earlier (*15*) was indicative of an improving health status. Since the time of the study, there have been indications that some of the measurements, particularly those for TSP, may have been subjected to a consistent negative bias in the range of 10 to 30% (*16*). For the current report, therefore, a comparison has been made of data collected by the USEPA with other data collected independently at the same or similar sites by the State of Utah.

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The study investigated a single hypothesis that the incidence or severity of acute respiratory illness was associated with the concentration of air pollutants to which populations were exposed. In addition, the effects of the more significant covariates on the illness rates were investigated. These include socioeconomic factors, smoking and the presence of chronic bronchitis.

Although analyses and presentation of these data have been delayed longer than usual, the information is relevant today because air quality standards continue to be a topic of controversy and because pressures to reduce the U.S. dependence on foreign sources of oil make it imperative that all information available relative to the possible impact of increased use of native coal supplies be reviewed.

Materials and Methods

Setting and Study Population

Four Utah communities, Magna, Kearns, Salt Lake City and Ogden, were selected for study on the basis of previously available aerometric and meteorologic data and similarity of socioeconomic status. The Utah setting was selected because past data suggested that a pollution gradient for SO_2 could be studied in a situation in which TSP was rather uniformly low. Magna was selected as the high pollution area, Ogden as the low pollution area and Kearns and Salt Lake City as intermediate areas. Families within each area were recruited from those responding to a chronic respiratory disease (CRD) questionnaire provided to every family that included an elementary school child. Since response was very good (approximately 95%), the candidate population was considered to be representative of families headed by younger adults with children attending elementary school. Each candidate family was contacted by an interviewer who completed a questionnaire concerning demographic characteristics whenever agreement for participation was given. Again, the refusal rate was low (estimated grossly by the investigators to have been <5%) and the sample enrolled was considered to be representative of that segment of the local population.

Interviewers, whenever possible, had had previous experience with telephone interviews. They were, in addition, given 2 or 3 days of intensive training to minimize interviewer bias. Interviewers were rotated routinely among the study subjects to maintain as much consistency as possible.

Approximately 250 families were recruited in each study area. The only restricting criteria when selecting families were that they must live within 1.5 miles (about 2.4 kilometers) of the air monitoring station; have lived in the home at least one year; have no plans to move within the subsequent year; have at least one child attending the local elementary school; and have a functioning telephone within the home. Families reporting substantial occupational exposure to dust by the head of the household also were excluded as were any individuals reported to have asthma, heart disease or chronic lung disease. These exclusions were made because of the potential confounding that could be associated with their inclusion.

The selection procedures limited the study populations to elementary school children and their immediate families. The incidence of illness was compared among these similar populations. It was not intended that the study populations should be representative of the total populations living in the various study communities.

Illness Monitoring

A telephone contact at biweekly intervals was made to each family to obtain information on illness experienced during successive two-week study periods. Specific information requested pertained to: the occurrence of any illness; fever; respiratory illness; restricted activity; and whether a physician had been contacted either in person or by telephone. If possible, the information was obtained from the mother or female guardian. If the mother was not available (estimated to have been < 2% of families, although not adequately documented), the information was obtained from whoever was considered to be the most responsible resident in the home.

Upper respiratory tract disease (URD) classification was given to illnesses whenever reported symptoms were limited to dry cough, head cold, sore throat, sinus or postnasal drip or runny nose. Lower respiratory disease (LRD) classification was given whenever the reported symptoms included chest cold, productive cough, asthma, croup, bronchitis or pneumonia.

The inquiry concerned asthma rather than wheezing because it was believed that among the population studied it would be better understood. Since individuals who on the initial questionnaire were identified as asthmatic were eliminated from the study, it is not believed that this question could have introduced a serious bias.

On a single occasion, a sample of the population was reinterviewed to determine the reliability of the data obtained by this telephone method. However, in no instance was confirmation by a physician obtained for the presence or absence of illness.

Monitoring Air Pollution Exposure

A single EPA air monitoring station was located in each study area. In Magna and Kearns the stations were atop one story buildings in 1971-72 but were moved to ground level at the same site for the 1972-73 study. In Ogden and Salt Lake City, the stations were at ground level during both years; however in both cities the monitoring site was shifted between 1971-72 study and the 1972-73 study a distance of about 0.5 miles to eliminate pollution generated by intense automobile traffic. Air monitoring data were collected during the same two year period by the Utah State Division of Health with monitors located at the 1971-72 EPA sites. These additional data provided a means of comparison in both years. At each station, 24-hr SO_2 measurements (West-Gaeke) and 24-hr high-volume particulate collections were made. Concentrations of water-soluble sulfates and water-soluble nitrates were obtained by laboratory analyses of the TSP collected. Nitrogen dioxide (NO_2) was monitored by the Jacob-Hochheiser method from 1970 to 1973.

Data Analysis

Data were analyzed for each year separately. Individuals reported on the initial interview to have asthma or heart or lung disease were excluded from the analyses, as were mobile families, defined arbitrarily as those living in the same residence for less than five years. Analyses were limited further to the white families since the percentages of nonwhites varied from area to area. Then only families who participated for one-half or more of the 15 study periods the first year or 16 study periods the second year were considered in the analyses. Comparisons were made among the three communities of illness rates per 100 person weeks of observations for fathers, mothers, and preschool (age 0-5 in 1971) and elementary (age 6-12 in 1971) school children. Repeated illnesses refer to separate events and not to continuing illness reported during two consecutive periods. Illness ratios were derived for each individual as:

$$\frac{\text{No. illnesses of a specific category reported}}{\text{No. weeks of participation}} \times 100 = \begin{array}{c}\text{Illness rate per}\\ \text{100 person-weeks}\end{array}$$

Each individual had three rates as disease indicators: upper respiratory illness rate, lower respiratory illness rate, and total respiratory illness rate. These individual illness rates were used as the dependent variable in multiple linear regression analyses.

Illness rates for groups were obtained by averaging the illness rates for all individuals within the group. Statistical significance of the group differences were determined by standard *t*-tests. The average severity of illness based on fever, number of days restricted activity, and the percent of illness prompting physician visits also were compared.

Other factors that might affect the incidence of respiratory illness were considered in the analyses. These included smoking habits of parents, crowding, educational status and bronchitis in parents. The importance of these factors was assessed by way of stratification and by the use of multiple linear regression analysis.

In the multiple regression analysis, a square-root transformation was made for the illness rates to meet more adequately the statistical assumptions. Analysis of variance tables were constructed to evaluate the effect of independent variables on the illness rates. Some interaction terms also were examined for their significance. These variables were tested by the sequential *F* test, with the community effect as the last variable to be entered in the model. The other variables were entered in the following order: education of the head of household, crowding, smoking, bronchitis, smoking + bronchitis and smoking + crowding.

The distribution of illness rates are inherently skewed to the left. However, the analysis of variance developed by the multiple regression method used is robust because a Fixed Effect Model was adopted and the sample sizes for the communities were large and approximately equal (*17, 18*). The square-root transformation reduced any effect due to tail-end cases.

Results

Air Pollution Exposures

Indications of the quality of the SO_2 and TSP data were obtained by comparing those collected by

EPA with those collected by the State of Utah. The comparison for SO_2 measurements suggested that while there were variations in the annual means determined, there were no consistencies with regard to the direction of the variations. Thus it was concluded that the approximate annual mean ambient concentrations of SO_2 were expressed equally well by either set of data. In addition, the moving of the EPA monitoring stations in Ogden and Salt Lake City did not effect significantly the level of agreement, although the differences decreased in Ogden and increased in Salt Lake City. In both Ogden and Salt Lake City, however, the measured levels were close to the minimum level detectable by the technique used.

Annual mean TSP values from the EPA monitors usually were lower than those determined by the state. The differences were greatest in 1971 and may represent a greater variability during the early phases of the EPA monitoring program. In all areas the annual means for 1972 from the EPA data were approximately 10 percent below those from the state. However, in 1973, the relocating of the EPA monitoring stations in Ogden and Salt Lake City to avoid the effects of high traffic densities had a significant effect on TSP measurements. Subsequent to the move, the annual mean TSP concentrations measured at the EPA monitoring sites in Ogden and Salt Lake City were 30 to 35% lower than those recorded by the state. Because the measurement best representing actual ambient exposure could not be determined, the higher value (state or EPA) for each community in each year was used to estimate exposure (Fig. 1). This presentation is least conservative and probably exaggerates the TSP exposures in Salt Lake City and Ogden.

The measured concentrations of SO_2 (West Gaeke method) showed a consistent difference across the study communities for the years 1971 to 1973 (Fig. 1) with Magna being high and the other communities relatively low. Daily data not presented in the figures show that the relative differences in exposure were consistent throughout the year. The 24-hr ambient air quality standard for SO_2 (240 $\mu g/m^3$) was never exceeded in Ogden or in Salt Lake City. The standard was exceeded infrequently during November, December, and January in Kearns and was exceeded with regularity during these months and infrequently during the remainder of the year in Magna.

Daily mean TSP concentration varied less. Measurements in excess of the 24-hr standard (260 $\mu g/m^3$) were recorded during the first or last quarter of the year in Ogden and Salt Lake City on more than 5% of the days, when collection were made adjacent to a busy roadway. The data indicated that TSP concentrations were highest in Salt Lake City and lowest in Kearns. After the EPA monitors in Ogden and Salt Lake City were moved to residential sites comparable to those in Kearns and Magna, both the annual and the quarterly data indicated that TSP concentrations were highest in Magna and lowest in Kearns. These data suggest that the annual mean concentration in the study area may have ranged from about 75 to 110 $\mu g/m^3$ and that the daily standard was exceeded rarely.

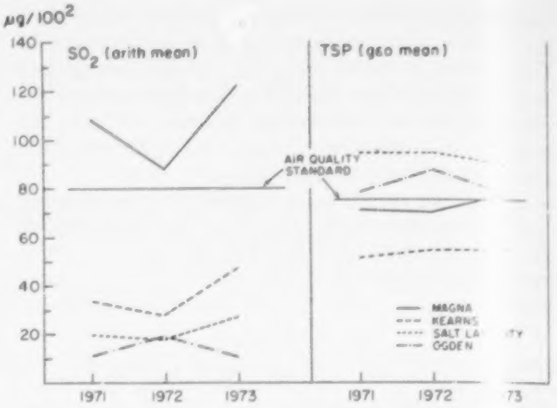

FIGURE 1. Concentrations of TSP and SO_2 in Utah study communities 1971-1973.

Nitrogen dioxide (NO_2) measurements by the Jacob-Hochheiser method are considered to be unreliable. However, in 1974 NO_2 was measured by the arsenite method, which gave the same relative concentrations among the study areas and almost the same numerical levels. These suggested that annual mean concentrations of NO_2 were only 0.2 to 0.5 the annual ambient air quality standard (100 $\mu g/m^3$), but that concentrations probably were highest consistently in Salt Lake City, nearly as high in Ogden and lowest in Magna and Kearns.

Annual mean suspended nitrate (SN) concentrations measured as water-soluble nitrates deposited on the high-volume filter were low (annual geometric means between 0.6 and 2.4 $\mu g/m^3$). However, during the months of December, January and February, 24-hr mean concentrations in excess of 15 $\mu g/m^3$ were measured with some regularity in all of the study areas, and the maximum 24-hr concentration for Ogden or for Salt Lake City was in the range of 50 $\mu g/m^3$.

Suspended sulfate levels also were low on the basis of annual means. Again, however, quarterly maxima and ninetieth percentile data indicate that, during the winter months, 24-hr mean levels in excess of 50 $\mu g/m^3$ were experienced in all areas

Table 1. Demographic characteristics of participants in studies of acute respiratory illness: Utah, 1971–1973.

Year		Crowding index[a]	Education[b]		Smokers, %	
			HS grad.	Coll. grad.	Mothers	Fathers
1971–72	Ogden	30	79	13	16	25
	Salt Lake City	34	72	13	15	26
	Kearns	42	73	8	20	30
	Magna	37	79	10	19	31
1972–73	Ogden	34	82	15	15	30
	Salt Lake City	39	75	16	20	29
	Kearns	45	83	8	22	31
	Magna	38	82	11	30	33

[a] Percent of families with more than one person per room in their home.
[b] Percent of heads of households with the indicated minimal attainment.

except Ogden, where lowest exposure levels occurred consistently.

Demographics and Personal Characteristics of the Study Populations

The demographic data collected (Table 1) indicated that there were some differences in the study populations based on educational attainment, crowding, parental smoking and race. These differences were controlled by limiting the analyses to white subjects and by the multiple regression.

The number of families from whom information was obtained for at least half of the study periods varied among the study communities for the 1971-72 study period from about 240 to 280 (Table 2). During the second study year, the same families comprised the major segment of the study population. However, there were some losses and some new recruitments for the second year of the study. Overall, the total study population for the second year was about 10 percent smaller than was that for the first year.

Participation for each family segment in each study community exceeded 95.9 percent in each year of study.

Illness Frequency and Severity

The reliability of data obtained through the telephone interviews was checked by reinterviewing families containing 1272 individuals. Comparison of the data obtained with those provided on the original interview several days earlier gave a concordance (i.e., consistent information for the two interviews) in excess of 96% with a reported illness rate of approximately 15%.

Incidence rates for respiratory illness during the study periods (Table 3) showed no consistency with the SO_2 pollution concentrations among the various family segments or between the two years of study. Mean severity scores showed no consistent relationships with the community of residence nor did the percentage of the population who experienced either no illnesses or multiple illnesses.

Table 2. Number of participants in studies of acute respiratory illness: Utah, 1971–73.

Year		All families				White stable families[a]			
		Ogden	Salt Lake City	Kearns	Magna	Ogden	Salt Lake City	Kearns	Magna
1971–72	Fathers	246	253	297	261	157	149	225	195
	Mothers	250	256	282	266	155	147	218	193
	School Children	433	453	574	577	265	252	442	437
	Preschool Children	156	180	245	206	89	84	188	157
	Totals	1085	1142	1380	1310	666	632	1073	982
1972–73	Fathers	222	230	240	251	137	131	199	187
	Mothers	221	225	246	258	135	133	196	194
	School Children	415	464	510	561	250	266	407	422
	Preschool Children	173	182	215	201	105	89	176	155
	Totals	1031	1101	1271	1271	627	619	978	958

[a] Stable families are those who have lived at the same address for at least 5 years.

**Table 3. Illness rates per 100 weeks of observation in families exposed to different concentrations of air polluti
1971–1973.**

	1971–72 study[a]			1972–73 study		
	Upper	Lower	Total illness	Upper	Lower	Tota
Fathers						
Ogden	2.72	1.18	3.90	2.93	0.91	
S.L.C.	3.54	1.07	4.61	2.51	0.77	
Kearns	3.26	1.24	4.50	3.13	1.00	
Magna	3.28	1.02	4.32	2.67	0.99	
Mothers						
Ogden	3.85	1.11	4.97	3.56	1.26	
S.L.C.	4.95	0.96	5.92	4.80	1.06	
Kearns	4.74	1.26	5.99	4.27	1.33	
Magna	4.64	1.64	6.28	3.91	1.38	
School Children						
Ogden	5.63	1.46	7.09	5.18	0.91	
S.L.C.	6.36	1.58	7.95	5.99	0.76	
Kearns	5.51	1.01	6.52	5.11	1.09	
Magna	4.94	1.18	6.13	4.94	1.12	
Preschool Children						
Ogden	7.35	2.36	9.72	7.40	1.96	
S.L.C.	8.14	2.81	10.95	7.75	1.60	
Kearns	7.77	2.21	9.97	6.67	1.79	
Magna	8.55	2.05	10.62	7.58	2.17	

[a] *t* tests of group differences for 1971–1972 gave $p = < 0.05$ for the following: (highest rate in italics) Fathers, upper, Ogde
Mothers, upper, Ogden vs. *SLC*, upper; Ogden vs. *Kearns;* all, Ogden vs. *Kearns;* lower, Ogden vs. *Magna;* all, Ogden v
lower, *SLC* vs. *Magna*. Schoolchildren, lower, *Ogden* vs. Kearns; all, *Ogden* vs. Magna; upper, *SLC* vs. Kearns; lower
Kearns; all, *SLC* vs. Kearns; upper, *SLC* vs. Magna; lower, *SLC* vs. Magna; all, *SLC* vs. Magna.

[b] *t* tests of group differences for 1972–1973 gave $p < 0.05$ for the following: Mothers, upper, *Ogden* vs. SLC. Schoolchildr
SLC vs. Kearns; lower, SLC vs. *Kearns;* upper, *SLC* vs Magna; lower SLC vs. *Magna*.

Neither the average number of days of restricted activity associated with illness or the number of physician visits showed a consistent pattern with area of residence, within the family segments, or from year to year.

A stratified analysis showed that smoking was related fairly consistently in both adults and their children with higher rates of LRD but not with higher rates of URD.

A similar stratification gave some evidence that children of parents with chronic bronchitis were themselves subject to higher rates of respiratory illness but the data were far from conclusive.

The regression analysis (Tables 4 and 5) supported an inconsistent association of illness rates with smoking or bronchitis and in addition indicated the educational attainment of the head of household was frequently significant. However, once the effect of these factors was removed, the adjusted rates showed little association with community of residence (Table 6). Usually, when statistically significant differences in illness rates were observed, the higher rate occurred somewhere other than the designated high pollution area, Magna. Since inclusion of the potential interaction factors added very little to
the analysis, they were not included in the
ments presented in Tables 4-6.

Discussion

The results of this study suggest that nei
incidence nor the intensity of reported res
illness was affected by exposure to mean
concentrations of SO_2 in the range of 12
above the present annual air quality standa
$\mu g/m^3$.

The results leading to this conclusion, h
could have been affected by factors not cont
this study but this is not believed to be
Possible confounders include the lack of i
dence among illness rates for the variou
members and the possibility that factors use
regression analyses may be multicollinear. T
ilarity of the relative risks of acquiring res
illness shown by the unadjusted rates in
and the adjusted rates in Table 5 suggests
combined effect of the potential confound
small.

The square-root transformation to devel

Table 4. Multiple linear regression rates of URD, LRD and total ARD on education, crowding, parental smoking, bronchitis in parents and residence among fathers, mothers, school children and preschool children: Utah 1971–1972 (white stable families only).

Main effect	df	F values		
		Upper	Lower	Total
Fathers				
Regression	7, 718	1.29	0.96	1.11
Education	1, 718	0.79	0.64	0.10
Crowding	1, 718	0.28	0.10	0.26
Smoking	1, 718	2.39	0.61	0.82
Bronchitis	1, 718	1.43	1.85	4.01[a]
Magna vs. Ogden	1, 718	2.78	0.58	0.58
Magna vs. SLC	1, 718	0.09	0.24	0.90
Magna vs. Kearns	1, 718	0.19	1.58	0.38
Mothers				
Regression	7, 705	2.99[a]	1.94	2.83[a]
Education	1, 705	4.97[a]	2.79	8.40[a]
Crowding	1, 705	0.19	0.96	1.51
Smoking	1, 705	4.01[a]	2.32	0.38
Bronchitis	1, 705	4.46[a]	0.42	4.47[a]
Magna vs. Ogden	1, 705	1.50	2.10	2.72
Magna vs. SLC	1, 705	3.02	5.15[a]	0.16
Magna vs. Kearns	1, 705	1.78	1.82	0.28
School children				
Regression	7, 1388	2.06[a]	4.47[a]	3.41[a]
Education	1, 1388	0.04	15.04[a]	2.96
Crowding	1, 1388	0.31	1.79	0.06
Smoking by parents	1, 1388	0.32	4.36[a]	0.03
Bronchitis in parents	1, 1388	3.37	0.00	4.00[a]
Magna vs. Ogden	1, 1388	3.27	1.02	4.08[a]
Magna vs. SLC	1, 1388	12.37[a]	4.49[a]	18.57[a]
Magna vs. Kearns	1, 1388	2.74	1.24	1.26
Preschool children				
Regression	7, 510	1.85	1.51	1.66
Education	1, 510	1.88	3.86[a]	4.59[a]
Crowding	1, 510	1.19	0.26	0.07
Smoking by parents	1, 510	2.92	2.35	0.49
Bronchitis in parents	1, 510	1.27	0.40	2.60
Magna vs. Ogden	1, 510	3.00	0.14	3.14
Magna vs. SLC	1, 510	0.40	3.10	0.16
Magna vs. Kearns	1, 510	3.14	0.48	2.60

[a] 7 df: $p < 0.05$, $F \geq 2.03$; 1 df: $p < 0.05$, $F \geq 3.86$.

ter distribution of illness rates for analysis would not be a valid procedure if there was wide variation in the number of weeks of participation by individual study subjects. However, the overall participation of nearly 96% was adequate to permit use of the method.

This study is not entirely inconsistent with the earlier study of CRD in the same communities which found significant increases among smokers and nonsmokers of both sexes after 4 to 7 years residence in the designated high pollution area (*19*). The few historic aerometric data available from the area indicate that, through the mid-1960s, the concentrations of particulate matter were 50% or more higher than they were in 1971, while the concentrations of SO_2 apparently varied less. It is possible therefore that the increased prevalence of adult CRD found in Magna in 1970 was due to prior extended exposure to higher concentration of both TSP and SO_2. More recent exposure to the same high SO_2 but lower TSP concentrations may not have been sufficient to produce higher rates of ARD. The prior higher concentration of both TSP and SO_2 might also have been the primary cause of increased illness rates found in the 1970 retrospective study of LRD in children (*15*).

Although the study results, in the opinion of the authors, suggest that SO_2 is a less significant pollu-

Table 5. Multiple linear regression rates of URD, LRD and total ARD on education, crowding, parental smoking, bronchitis in parents and residence among fathers, mothers, school children and preschool children: Utah 1972–1973 (white stable families only).

Main effect	df	*F* values Upper	 Lower	 Total
Fathers				
Regression	7, 646	1.11	0.96	1.35
Education	1, 646	1.57	1.70	0.27
Crowding	1, 646	1.33	0.49	2.21
Smoking	1, 646	0.44	0.65	1.28
Bronchitis	1, 646	0.00	2.28	0.63
Magna vs. Ogden	1, 646	0.42	0.01	0.24
Magna vs. SLC	1, 646	0.52	1.46	1.80
Magna vs. Kearns	1, 646	1.69	0.01	0.85
Mothers				
Regression	7, 650	2.38[a]	1.11	1.02
Education	1, 650	0.17	0.29	0.34
Crowding	1, 650	4.95[a]	0.33	3.98[a]
Smoking	1, 650	4.01[a]	4.68[a]	0.36
Bronchitis	1, 650	0.03	0.76	0.25
Magna vs. Ogden	1, 650	0.81	0.04	0.44
Magna vs. SLC	1, 650	3.19	0.25	0.88
Magna vs. Kearns	1, 650	0.29	0.09	0.01
School children				
Regression	7, 1337	1.73	2.63[a]	1.31
Education	1, 1337	1.14	0.56	1.22
Crowding	1, 1337	1.27	2.16	2.75
Smoking by parents	1, 1337	0.75	6.63[a]	0.07
Bronchitis in parents	1, 1337	2.71	1.23	1.78
Magna vs. Ogden	1, 1337	0.56	1.40	0.02
Magna vs. SLC	1, 1337	7.53	2.66	3.14
Magna vs. Kearns	1, 1337	0.76	0.00	0.47
Preschool children				
Regression	7, 517	1.12	0.79	1.49
Education	1, 517	0.13	1.47	0.57
Crowding	1, 517	1.12	0.83	2.41
Smoking by parents	1, 517	0.01	0.37	0.30
Bronchitis in parents	1, 517	3.01	0.07	3.57
Magna vs. Ogden	1, 517	0.04	0.22	0.33
Magna vs. SLC	1, 517	0.00	3.16	1.02
Magna vs. Kearns	1, 517	2.42	1.38	4.62[a]

[a] 7 df: $p < 0.05$, $F \geqslant 2.03$; 1 df: $p < 0.05$, $F \geqslant 3.86$.

tant than is TSP at the levels monitored during this study, it is not possible from the data collected to indicate safe or unsafe concentrations of either pollutant numerically. This is due to the inaccuracies associated with pollutant measurements; the inability to determine whether short-term or long-term exposure is the more important criterion; the lack of knowledge concerning possible interactions between a number of pollutants; the inability to associate individual exposures with the data collected at single stationary air pollution monitors; and the lack of information concerning the possible presence of other toxic air pollutants. For example, in two of the Utah study communities, the measurements of TSP were affected significantly by the proximity of a heavily traveled roadway to the air monitored stations. This would tend to overestimate TSP exposure in the quieter residential areas even though accurate measurements were made. In addition, it has been shown that the West-Gaeke method for collecting SO_2 is temperature-dependent ([illegible]0), a fact that may have affected measurement in Utah very little during the winter months, but the total effect on annual means is unknown. Any effect would have tended to produce underestimations of actual SO_2 exposures. For these reasons, the actual TSP exposures may have been even more similar in the four study communities than indicated in Figure, 1 and the

Table 6. **Relative rates of ARD in families in Utah study areas adjusted for education, crowding, parental smoking, and bronchitis in parents and area of residence (white stable families only): 1971–1972 and 1972–1973.**

Year		Upper	Lower	Total
1971–1972	Fathers			
	Ogden	0.83	1.18	0.94
	SLC	1.13	1.08	1.11
	Kearns	1.02	1.16	1.07
	Magna	1.00	1.00	1.00
	Mothers			
	Ogden	0.80	1.25	0.78
	SLC	1.14	0.64[a]	0.98
	Kearns	1.07	0.78	0.98
	Magna	1.00	1.00	1.00
	School children			
	Ogden	1.15	1.25	1.18
	SLC	1.29[a]	1.38[a]	1.32[a]
	Kearns	1.11	0.86	1.08
	Magna	1.00	1.00	1.00
	Preschool children			
	Ogden	0.80	1.06	0.88
	SLC	0.89	1.22	0.99
	Kearns	0.86	1.01	0.91
	Magna	1.00	1.00	1.00
1972–1973	Fathers			
	Ogden	1.11	0.97	1.06
	SLC	0.93	0.83	0.89
	Kearns	1.19	1.00	1.14
	Magna	1.00	1.00	1.00
	Mothers			
	Ogden	0.86	0.98	0.91
	SLC	1.27	0.86	1.12
	Kearns	1.10	0.99	1.06
	Magna	1.00	1.00	1.00
	School children			
	Ogden	1.06	0.88	1.02
	SLC	1.24	0.79	1.13
	Kearns	1.05	0.98	1.04
	Magna	1.00	1.00	1.00
	Preschool children			
	Ogden	0.93	0.90	0.93
	SLC	0.97	0.74	0.91
	Kearns	0.83	0.81	0.73[a]
	Magna	1.00	1.00	1.00

[a] Rate is significantly different ($p < 0.05$) when contrasted with Magna, the designated high pollution area.

annu l mean SO_2 exposure, particularly in Magna, may ave been higher than indicated in Figure 1.

In oor pollution, particularly NO_2 associated with sing a gas stove for cooking, has been shown in so ne areas to be associated with higher rates of respi atory illness (*21*). Such pollution may have been a significant factor in Utah but appropriate data or determining such significance were not colle ted. NO_2 also may have been an important ambi nt pollutant in this study, especially in the desig ated low pollution areas.

In onclusion, it is believed that the data support the h pothesis that SO_2 in high concentrations apparently had little or no effect on the incidence of acute respiratory illnesses in Utah.

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Environmental Health Perspectives
Vol. 44, pp. 175-188, 1982

Toxicity of Polybrominated Biphenyls (PBBs) in Domestic and Laboratory Animals

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The composition, environmental fate, and effects of the polybrominated biphenyls (Firemaster BP-6 or FF-1) involved in the accidental contamination of cattle feed in Michigan in 1973 are reviewed. Toxic effects referred to in this report are limited to those occurring in domestic and laboratory animals and include general toxicity, neurobehavioral toxicity, immunotoxicity, reproductive toxicity, mutagenicity and carcinogenicity. The absorption, distribution, biotransformation and elimination of these polybrominated biphenyls are discussed along with the interactions with other chemicals and drugs.

Introduction

Polybrominated biphenyls (PBBs) have been widely used as flame retardants in industrial and consumer products. In 1973, PBBs accidentally entered the food chain in Michigan, when Firemaster FF-1, a commercial flame retardant, was inadvertently substituted for magnesium oxide used as a supplement in the formulation of cattle feed (*1*). Ten to twenty bags, 50 lb each, of PBBs were mixed into feeds that were widely distributed to Michigan farmers, and consumed by a large number of farm animals. Three feed preparations containing different concentrations of PBBs appeared initially to be involved Feed No. 405, 2.4 ppm;† No. 410, 1790 ppm; and No. 07, 4300 ppm (*2*). A concentration as high as 13,500 ppm of polybrominated biphenyls was also cited (*3*). Dairy cattle were the first to show signs of intoxication (decreased milk production, anorexia, alopecia, abnormal growth) which were attributed to the ingestion of PBB-contaminated feed. Subsequently, other farm animals were also found to have been contaminated and adversely affected. For about 9 months following the accident, contaminated dairy and poultry products were consumed by Michigan farmers and residents. In order to minimize further human exposure, at least 29,800 cattle, 5,900 hogs, 1,500 sheep, and 1.5 million chickens were destroyed along with large amounts of animal feed, cheese, butter, dry milk products and eggs (*4*).

Chemical Composition

The Michigan Chemical Corporation produced Firemaster FF-1 by adding an anticaking agent, calcium polysilicate (2%) to a commercial flame retardant known as Firemaster BP-6. Firemaster BP-6 is a mixture of polybrominated biphenyl compounds, containing 2-8 bromine atoms (*5*). Table 1 shows the major components of Firemaster BP-6 analyzed by gas chromatography combined with mass spectrometry. The major constituents of Firemaster BP-6 are 2,4,5,2′,4′,5′-hexabromobi-

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† 1 ppm = 1 mg/kg of feed or diet.

Table 1. Brominated compounds related to Firemaster FF-1 (BP-6).

Reported components	Concentration	Refe ce
2,2′-Dibromobiphenyl	200 ppm	(9
Tetrabromobiphenyl(s)	2%	(1
2,4,5,2′,5′-Pentabromobiphenyl }	10%	(1
2,4,5,3′,4′-Pentabromobiphenyl }	8%	(1
	4%	(
Hexabromobiphenyl	56%	(
2,4,5,2′,4′,5′-Hexabromobiphenyl }		
2,3,4,2′,4′,5′-Hexabromobiphenyl }	63%	(6,)
2,4,5,3′,4′,5′-Hexabromobiphenyl }	{ 14%	(
Two heptabromobiphenyls	{ 33%	(
2,3,4,5,2′,4′,5′-Heptabromobiphenyl	27%	(
Octabromobiphenyl }	11%	(
2,3,4,5,2′,3′,4′,5′-Octabromobiphenyl }		
Unknowns		
Tetrabromonaphthalene(s)		(7,)
Pentabromonaphthalene(s)	150 ppm	(
	1 ppm	(
Hexabromonaphthalene(s)	70 ppm	(
	25 ppm	(

phenyl (56%) and 2,3,4,5,2′,4′,5′-heptabromobiphenyl (27%) (*6, 7*). Firemaster BP-6 was also found to be contaminated with low concentrations (0.02% or less) of penta- and hexabrominated naphthalenes (*6, 8*). Firemaster BP-6 was analyzed for the presence of polybrominated dibenzo-*p*-dioxins and polybrominated dibenzofurans because of structural homology to their known very toxic chlorinated derivatives (Fig. 1). If present, the concentrations were less than 0.5 ppm (*6*). Phenoxyphenols and hydroxybiphenyls, which might be intermediates in the formation of brominated dibenzo-*p*-dioxins and brominated dibenzofurans, respectively, were not identified (*8*).

Small amounts of PBB metabolites and decomposition products have also been detected by chromatographic and spectrometric methods (*13-18*). Several of these are listed in Table 2. Brominated dibenzofurans can be formed by thermal degradation of PBBs. Ultraviolet degradation cleaves bromine atoms leading to several debrominated and oxygenated biphenyl products (Table 2).

Fate of PBBs in the Environment

Because of their stability, PBBs may remain in the environment for a long time, raising questions about their transport and degradation in such environmental components as soil, water, plants, and animals.

The Michigan soils have been contaminated with PBBs from the manure of contaminated animals and from the disposal of feed, milk, carcasses, dust cleanings, etc. Once PBBs have been introduced into the soil, they appear to have little tendency to translocate. A study on the ability of rainfall to

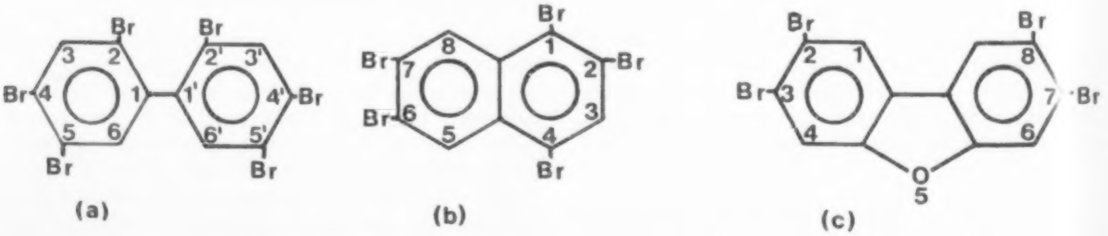

FIGURE 1. Representative polybrominated (a) biphenyl, (b) naphthalene, (c) dibenzofuran.

Table 2. Metabolites and decomposition products of polybrominated biphenyls.

Compound	Occurrence	Reference
Metabolites		
6-Hydroxy-2,4,5,2′,4′,5′-hexabromobiphenyl	Metabolite in dog feces	*(13)*
Pentabromobiphenylol	Metabolite in pig urine and/or feces	*(14)*
Several mono- to pentabromobiphenyls that are not present in BP-6	Metabolites present in fish	*(15)*
Thermal decomposition products		
1,3 ,7,8-Pentabromodibenzofuran 1,3 ,6,7-Pentabromodibenzofuran	Decomposition products of 6-hydroxy-2,4,5,2′,4′,5′-hexabromobiphenyl in gas chromatograph at 230-260°C	*(13)*
2,3 ,8-Tetrabromodibenzofuran Pentabromodibenzofuran	Decomposition products of Firemaster FF-1 by open tube pyrolysis at 380-400°C	*(16)*
Ultraviolet decomposition products		
2,4 ,3′,4′-Pentabromobiphenyl 2,4 ,2′,4′-Pentabromobiphenyl Tetrabromobiphenyl(s) Methoxylated product Dimethoxytetrabromobiphenyl	Decomposition products of Firemaster BP-6 at 2537 or 3000 A	*(17, 18)*

carry PBBs through the soil involved a laboratory simulation which analyzed only the major component of Firemaster, hexabromobiphenyl. This study indicated that hexabromobiphenyl was retained in the top soil and did not leach further (*19*). Although laboratory studies indicated that PBBs can be degraded by ultraviolet light (*20*), no significant degradation occurred in the field even after a year, and, what little degradation did occur, resulted in products with a much greater affinity for soil than that of parent compounds (*21*). Therefore, degradation by ultraviolet light is only a minor decontamination process for PBBs (*22*), affecting the small amount of PBBs on the soil surface. PBBs also appear to be fairly resistant to microbial degradation in soil (*23*).

Because of the apparent persistence of PBBs in soil, several studies have been done to determine whether forage (orchard grass, etc.) and root crops (onions, radishes, carrots, etc.) might take up these contaminants and reintroduce PBBs into the food chain (*21, 23, 24*). Both laboratory and field studies indicate that there was no uptake of PBBs into the plant foliage (*21, 23*), but that trace amounts of PBBs were associated with the root surfaces of edible root crops (*24*). Recontamination of animals from plants grown on contaminated soil is not likely to occur. No degradation of PBBs by plants has been recorded.

Water sampling in the Pine River downstream from the Michigan Chemical Corporation showed detectable levels (0.01-0.07 μg/l.) of PBBs in 1974 (*25*). River sediments also contained detectable levels (100-6200 μg/kg) of PBBs. No degradation of PBBs in water has been reported, although the possibility of photodegradation at the water-air interface exists. With the exception of the heptabromobiphenyls, the PBBs in water are readily taken up by fish and accumulated in their tissues (*15*). As an example of the efficiency of this bioaccumulation, fish living in water containing less than 0.1 μg PBB/liter of water concentrated these contaminants in their bodies 10,000-fold within two weeks (*25*). Fish are capable of metabolizing PBBs, but not to a degree sufficient for any significant degradation (*15*). Ducks living near contaminated waters also accumulate PBBs in their adipose tissue (*25*). Follow-up surveys since the termination of Firemaster BP-6 production show no significant decline of PBB levels in river sediments and fish and duck tissues. Thus, PBBs are likely to remain an environmental problem for a long time.

Absorption, Distribution, Biotransformation and Elimination of PBBs

An understanding of the true biological behavior of PBBs in the form of Firemaster BP-6 is complicated by the nature of the commercial product which is a mixture of compounds whose biological activity differs according to the position of bromine in the molecule and to a lesser degree by the number of bromines in the molecule (*26-29*).

A number of experiments have been performed in cows, pigs, rats, and birds on the absorption, biotransformation, tissue distribution, and excretion of the Firemaster BP-6 and several of its major components. These studies indicate that PBBs are rapidly and almost completely absorbed (absorption apparently decreases with increasing bromine content) from the gastrointestinal tract and are initially distributed widely throughout the body tissues with major concentrations in liver and fat (*1, 5, 29-44*). Based on the available data, the PBBs in Firemaster BP-6 are apparently metabolized to give hydroxylated degradation products by metabolic routes that are similar to those for the structurally related polychlorinated biphenyls (*14, 45-48*).

Pharmacokinetic studies in rats have revealed that the individual PBBs in Firemaster BP-6 are eliminated from the body at different rates (*26-29*). As a result of this differential rate of elimination, those components that are more slowly removed seem to become more concentrated relative to the other PBBs (*28*). The rates of metabolism appear to be determined primarily by the location of the bromine in the biphenyl molecule and secondarily by the bromine content or the availability of two adjacent unsubstituted sites in the biphenyl molecule (*26-29*). Similar relationships have been reported for the PCBs (*48, 49*). The major technical hexabromobiphenyl component, 2,4,5,2′,4′,5′-hexabromobiphenyl, appears to be the most persistent component in the species studied (*26, 27, 43*).

Excretion of PBBs is primarily via the bile into feces. Additional excretory routes for these chemicals are via lipophilic secretions such as hair and skin oil. Milk and eggs are the major routes in lactating mammals and egg-laying birds, respectively (*43*). Transplacental transfer of PBBs has been demonstrated in rats and cows and detrimental effects in young suckling on PBB-contaminated mothers have been reported (*5, 29, 37, 40-42*).

A number of metabolic studies of PBBs have been performed in cows. In one of the largest experiments to date, sixty dairy cows at nearly all stages of development and productive life were given daily doses of 0.25 mg to 25 g of Firemaster BP-6 for 1 to 202 days and were followed experimentally from 10 days to 3 years (*29*). It was found that the major (hexabromobiphenyls) and minor (pentabromobiphenyls, heptabrombiphenyls) brominated biphenyls of the commercial mixture were absorbed from the gastrointestinal tract and appeared in the blood plasma within 4 hr. With continued exposure, a steady state for the chemical in the plasma was reached after 15 days. PBBs had a predilection for high lipid-containing tissues of the cows and similar concentrations were found in the various fat depots. However, PBB concentrations were low in the nervous system despite its high lipid content and disproportionately high in liver considering the relatively low lipid content of that organ. In cows not showing signs of toxicity, the major route of elimination, representing 50% of the amount of PBBs fed, was the feces. Nonmetabolized PBBs were not detectable in the cows' urine, although this route of excretion may account for minor or metabolized brominated biphenyls. Following withdrawal of PBBs, the fecal concentration declined to 1–2% of fecal levels during dosing but feces remained the major elimination route in non-lactating animals. Milk fat was the major route of excretion in lactating animals, accounting for three times the quantity of PBB residue eliminated in feces. The half-time of PBB excretion via the maternal milk was 28 days. PBB concentrations in milk declined approximately twofold in six days following birth of young. Following parturition the PBB residues remained at a level of about 0.[illegible]% of that in the depot fat. Calves born to PBB-exposed cows had distributions of PBB residues in body tissue similar to those in the mothers but the concentrations were lower (*29*). This study and others (*1, 30, 36, 37*) have demonstrated that growth, fat deposition, fat metabolism, lactation, pregnancy and level of exposure can modulate the biotransformation of PBBs and that simple kinetic models are inadequate to describe the long-term behavior of PBBs in cows.

To the limited extent that they have been studied, the patterns of metabolism and tissue distribution in the pig, guinea pig, and rat are comparable to that in the cow (*5, 26-28, 33, 40, 47, 49*). However, in the rat, unlike the cow, the heptabromobiphenyl component was retained in significant proportions 42 days after administration (*26*). In hens, elimination of PBBs through the egg is more important than elimination through the excreta or storage in body fat (*34, 38*). The level of PBBs in eggs has been reported as about 1.5 times the dietary levels (*34*). After 63 days of dietary exposure to PBBs, the concentration of these chemicals in body fat of chickens was about four times that in the diet (*34*). The PBB residues in female egg-laying quail were generally lower than in males of the same species (*39*).

The kinetics of several individual components of Firemaster BP-6 have also been studied. When the major component, 2,4,5,2′,4′,5′-hexabromobiphenyl, was fed to male rats at a dose of 1 mg/kg, as single or multiple doses, 90% was absorbed from the gastrointestinal tract. This compound was distributed throughout the body with predominant initial localization in muscle, fat, and skin. The chemical was extremely resistant to metabolism. It was estimated on the basis of the short-term metabolic studies that only 9.5% of the total dose would ever be excreted in the feces. These findings suggested that male and nonnursing females and non-egg-laying birds would be unable to effectively clear their tissues of ingested PBBs in their lifetimes (*43*). There is evidence that another PBB component, heptabromobiphenyl, disappears more quickiy from milk (*36*) and eggs (*38*) than the hexabromobiphenyl described above.

Finally, metabolic studies of "octabromobiphenyl" (OBB), which consists of a mixture of hepta-, octa-, nona- and decabromobiphenyl averaging approximately eight bromine atoms per molecule, provide an insight into the disposition of the octabromobiphenyl component of Firemaster BP-6 that represents about 11% of the commercial mixture. This mixture, when fed to rats at 1000 ppm for 4 weeks, produced bromine concentrations in the fat that were approximately 600 times greater than the bromine levels in the fat of nontreated animals. Eighteen weeks after OBB was removed from the diet, the fat bromine residues had continued to increase and were 800 times greater than the bromine levels in the fat of control animals, implying that these compounds are retained in the tissues over a prolonged period. However, no comparisons with hexabromobiphenyl retention were possible from these studies (*44*).

Enzyme Induction by PBBs

Basic biochemical studies in laboratory rodents have shown that Firemaster BP-6 is a potent inducer of a group of liver (*50-56*) and kidney (*57*) microsomal enzymes, identical to those induced by phenobarbital and those induced by 3-methylcholanthrene (*51, 52*). Phenobarbital-type enzyme inducers increase the concentrations of cytochrome P-450, NADPH-cytochrome c reductase, and a wide range of microsomal enzymes whereas 3-methylcholanthrene-type inducers increase the concentration of cytochrome P_1-450 and stimulate a more specific group of enzyme activites not including the reductase (*58*). While phenobarbital-like microsomal enzyme stimulators also enhance bile flow and the biliary excretion of drugs, 3-methylcholanthrene-like inducers do not produce such effects (*59, 60*).

Commercial mixtures of polychlorinated biphenyls have produced a similar mixed type of induction (*61*) which has been attributed to the different inducing capabilities of individual components of the mixture (*62*). Thus far, two components have been identified. They are 2,4,5,2′,4′,5′-hexabromobiphenyl and 2,3,4,5,2′,4′,5′-heptabromobiphenyl, which together comprise 83% of the commercial mixture (*54*). Constituents of Firemaster BP-6 that are responsible solely for the 3-methylcholanthrene-type enzyme induction are unknown (*54-56*). Since the toxicity of several polyhalogenated biphenyls appears to be correlated with an ability to induce enzymes like those induced by 3-methylcholanthrene (*63, 64*), identification of such components might help to better characterize the toxicity and mechanism of action of PBBs in the commercial mixture. Indirect evidence for the presence of such components comes from a study in mice which revealed that the liver enzymes induced within 24 hr of exposure to Firemaster BP-6, were equivalent to those induced by phenobarbital, whereas those present 96 hr later were the same as the enzymes stimulated by 3-methylcholanthrene (*51*), implying a sequential metabolism of components with phenobarbital and 3-methylcholanthrene-like inducing properties.

In tests of the potency of the enzyme-inducing capability of Firemaster BP-6, the commercial mixture produced minimal enzyme induction at a dose level of 0.3 mg/kg/day and maximum induction at 30 mg/kg/day (*54*). Mixed induction was produced via both the transplacental route and the milk (*65, 66*). Lactating rats exposed to a dietary level of 1 ppm of Firemaster BP-6 showed no significant enzyme induction, whereas several enzymes were induced in nursing young at this dose level (*67, 68*).

Chemical Interaction of PBBs with Other Chemicals and Drugs

The ability of PBBs to induce a wide range of microsomal enzymes in liver and kidney involved in activation and deactivation of foreign compounds implies that PBBs have a high capability for altering the biologic activity of a variety of chemicals and drugs (*69, 70*). Several experimental studies support this assumption. It has been shown that mice ingesting PBBs in the feed for two weeks (as Firemaster BP-6, in concentrations of 1, 20, 25 or 100 ppm) developed an increased susceptibility to the renal and hepatic lesions induced by single intraperitoneal injections of the chlorinated hydrocarbon solvents, chloroform and carbon tetrachloride (*71*). As little as 0.025 ml/kg of chloroform produced abnormalities in liver and kidney function. While a dose of 0.005 ml/kg of carbon tetrachloride caused similar changes in the livers of these mice, 0.125 ml/kg of the solvent was required to produce kidney changes. Kidney changes were also observed in the PBB-treated mice when they were given 1.0 ml/kg trichloroethylene or 0.15 ml/kg or 1,1,2-trichloroethane. Since PBB administration stimulates microsomal enzyme systems in liver and kidney that appear to be necessary for the generation of toxic metabolites from these chlorinated hydrocarbon solvents, it can be assumed that the mechanism of PBB-induced potentiation of solvent toxicity is stimulation of a particular enzyme or enzyme pathway resulting in the generation of greater amounts of toxic metabolite in PBB pretreated mice. These results imply that long-term exposure to PBBs might also sensitize humans or animals to the toxic action of chemicals requiring biotransformation (*71*).

There is also limited evidence that enzyme induction by PBBs may decrease the biological activity of some chemicals. Female rats maintained for approximately one year on a diet containing 50 ppm PBBs, as Firemaster BP-6, and 300 ppm N-2-fluorenylacetamide (2-FAA), a potent carcinogen, lived longer and developed 50% fewer tumors of the ear duct and mammary glands than did animals fed only 2-FAA in the diet. The PBB supplement also appeared to prolong the time to appearance of these tumors (*72*). Since the chemical 2-FAA requires metabolic change to the active carcinogen, it is possible that the enzymes induced by PBBs may have converted the 2-FAA mainly into inactive metabolites and thus reduced the amount of carcinogen bound to DNA (*73*, *74*). Further studies are needed to verify this hypothesis.

The results of a recent experiment imply that PBBs may also affect the activity of chemicals by influencing their excretion without affecting their biotransformation (*58*). In this study, dietary exposure of mice to Firemaster BP-6, enhanced the disappearance from plasma of two drugs, indocyanine green and ouabain, which do not require biotransformation for their activity. At 10 and 15 min following the intravenous injection of 40 mg/kg indocyanine green, the plasma levels of the drug were lower in animals that had been fed PBBs in the diet at levels of 100, 150, and 200 ppm than in those controls receiving no PBBs. The plasma concentration of ouabain given intravenously at 0.1 mg/kg was also much lower after 60 min in mice fed 100 and 200 ppm PBB than in controls. PBB-fed mice showed an enhanced uptake of both drugs by the liver, and an increase in liver weight. These effects appeared to be similar to those observed after phenobarbital pretreatment at 75 mg/kg/day for 4 days. Since indocyanine and ouabain are not metabolized prior to excretion into the bile, their enhanced disappearance from plasma cannot be explained by an altered rate of biotransformation. As was the case with phenobarbital pretreatment, however, the increased rate of disappearance from plasma was associated with an increased liver mass. Additional factors, not yet explored for PBBs, but important to the phenobarbital effect, are increases in hepatic blood flows and bile flow (*58*).

Finally, a further example illustrates the range of biological effects that can result from the interactions of PBBs with other chemicals in the body. It has been shown that a dose of 200 mg/kg of PBBs in the form of Firemaster BP-6 increased iron transport in the rat intestine by 50-60% as determined in everted duodenal gut sacs (*75*). The effect of PBB on iron transport was biphasic; iron transport was maximal two days after treatment and returned to normal levels two days later, but increased significantly to about 20% more than in controls at 7 and up to 18 days after treatment. This effect on iron absorption could be elicited by a dose as small as 2.5 mg/kg PBB without any overt signs of intoxication or weight loss. This experimental finding is corroborated by an increased serum iron content in cattle accidentally exposed to PBBs on farms in Michigan. Whether increased absorption is the sole mechanism for the increases in serum iron, however, is unknown, and further studies on the effect of chronic exposure to PBBs on iron absorption and total body iron are necessary (*75*).

General Toxicity of PBBs

Until the Michigan accident, very little was known about the toxicity of PBBs. Toxicological assessments of Firemaster FF-1 have been complicated because this commercial preparation contains a mixture of PBBs as well as traces of impurities (brominated naphthalenes) which have their own toxic properties. Adverse effects may also vary with the species exposed, the age of the animal, and the duration and dose of exposure. Gupta and Moore (*76*) showed that PBBs are more toxic when given in small repeated doses versus a single acute dose. In most acute and chronic feeding studies, signs of PBB toxicity include weight loss or a decreased weight gain, and an increase in liver size (*76-85*). Weight loss is not necessarily accompanied by decreased food intake (*76*, *81*) suggesting that PBBs may cause poor feed utilization.

Several studies on PBB toxicity in rats found that concentrations as low as 50 ppm (Firemaster BP-6) in the diet of male rats for 10 weeks produced an enlarged liver (*77*). Female rats fed 100 ppm Firemaster BP-6 for 90 days, showed retarded weight gain and altered activity of several kidney enzymes, but no loss of kidney function (*78*). Rats given 30, 100, 300 and 1000 mg/kg/day (5 days/week, 22 total doses) of Firemaster FF-1 by stomach tube were observed for 180 days after the beginning of treatment (*76*). All the animals exposed to dose rates greater than 30 mg/kg/day died within 73 days, with the exception of 62% of the males at 100/mg/kg/day which survived 180 days until sacrifice. All surviving rats demonstrated decreased body weight gain (accompanied by significant decreases of food consumption), anemia, and enlarged livers. Histopathologic studies showed differences between the toxicities of PBBs to surviving male and female rats. Liver, kidney, prostate and thyroid glands were primarily affected in male rats, whereas only the liver was altered in female rats. The animals which became moribund or died early had marked atrophy of the thymus and spleen (*76*).

Mice fed 1000 ppm of Firemaster BP-6 in the diet for 4, [illegible], 11 and 14 days showed progressive signs of intoxication, body weight loss, and enlargement of the liver (*79*). Electron microscopy of liver tissue revealed increases in smooth endoplasmic reticulum and lysosomes, mitochondrial degeneration and decreases in glycogen.

Chickens fed Firemaster FF-1 in the diet at levels ranging from 50 to 250 ppm demonstrated the following adverse effects which were accentuated by increasing dose and/or length of exposure: decreased body weight due to decreased feed intake, decreased comb, testes, spleen, and bursa weights, increased liver and thyroid weights, hydropericardium and ascites (*80*). In another study, chicks fed 75 or 150 ppm of Firemaster FF-1 in the diet for 9 weeks showed cardiovascular and hematological effects which included decreased heart rate, cardiac output, packed cell volume, and hemoglobin concentration. This anemic condition was thought to be due to both decreased erythropoietin production essential for red blood cell formation by the kidney and to a direct action on the bone marrow (*80*).

Nonhuman primates experimentally exposed to Firemaster FF-1 also showed anemia which may have been due to decreased erythropoietin production (*81*). Seven female rhesus monkeys fed 0.3 ppm in the diet of Firemaster FF-1 for approximately 2 years showed reduced body weights for 6 months despite unmodified food intake, and prolonged menstrual cycles and decreased progesterone levels (*81*). Three monkeys fed 1.5 ppm for 38 weeks had a moderate weight loss, moderate fatty infiltration of the liver and decreases in serum cholesterol, while two monkeys on a diet containing 25 ppm for 14 weeks developed hyperplastic gastroenteritis (*82*). This hyperplastic gastroenteritis and the accompanying ulcerations were the most severe and acute lesions that were observed in PBB-exposed monkeys. Additional signs seen at 25 and 300 ppm PBB included enlarged livers, dry scaly skin, hair loss, generalized subcutaneous edema and marked edema of the eyelids (*81*).

Dairy heifers fed 250 mg/day of Firemaster BP-6 for 2[illegible] days, showed no clinical evidence of hepatic, renal or intestinal toxicity (*83*). But dairy heifers fed 25 g/day of Firemaster BP-6 for 33-60 days showed weight loss, dehydration, atrophy of the thymus, and liver and kidney enlargement (*84*, *85*). Histopathological and microscopic changes were most pronounced in the kidneys, gall bladder, and eyelids. In calves, a dose rate of 100 mg/kg/day of Firemaster FF-1 in the diet for 2-12 consecutive weeks produced histopathological lesions in the kidney, liver, skin, and testes (35).

In order to evaluate the comparative toxicity of PCBs and PBBs, adult male rats were fed 0, 5, 50, 500 ppm Aroclor 1254 (PCBs) or Firemaster BP-6 (PBBs), in the diet, for 2, 3 or 5 weeks. Firemaster BP-6 tended to cause equivalent adverse effects at lower doses, or in shorter time periods or had a greater effect at the same dose level than Aroclor 1254. The effects which were more pronounced with Firemaster BP-6 were an enlarged liver, an increase in liver cholesterol and plasma cholesterol and a decrease in liver RNA synthesis. Firemaster BP-6 was also found to be about three times more potent than Aroclor 1254 in inducing microsomal enzyme activities (*86*).

Neurobehavioral Toxicity of PBBs

Although clinical studies of farmers exposed to PBBs in Michigan have revealed a high incidence of neurologic and behavioral signs and symptoms (*87*), relatively little information is available from experimental studies concerning the effects of PBBs on the nervous system.

Tilson and Cabe (*88*) used a battery of tests to determine whether known amounts of PBBs given to laboratory rats and mice resulted in adverse behavioral and neurological effects. Animals were given doses of FF-1 or 2,4,5,2′,4′,5′-hexabromobiphenyl (HBB). Neurobehavioral toxicity was assessed at the end of a 30-day dosing regimen, and also 30 days after cessation of dosing in order to determine the delayed onset of signs or the duration of any effects noted. These investigations indicated that PBBs given orally to mice and rats reduced body weights, decreased motor activity, depressed motor reflexes, and impaired forelimb grip strength. Such effects were noted in rats receiving a total dose of 100-150 mg/kg of FF-1 during 30 days of dosing. The effects in rats tended to worsen over the 30 days after treatment, but those in mice tended to improve. HBB, the major component of the FF-1 mixture, was less toxic than the FF-1 mixture. Performance on tests sensitive to detecting peripheral neuropathy and emotionality changes was not affected by doses of FF-1 up to 1300 mg/kg. These authors concluded that the neuromuscular weakness and behavioral depression seen in laboratory animals after repeated exposure to PBBs are relatively nonspecific, and may be due to the effects of PBBs on organ systems other than the nervous system.

In another study (*89*), however, PBB (FF-1) levels were still detectable in the brains of rats 10 months after the last of a series of twenty 1.0 mg/kg doses. In a related study (*90*), rat tissue concentrations of PBBs, measured under conditions of weight loss severe enough to cause the disappearance of body fat, continued to exhibit a marked dose-response relationship with respect to retention of PBBs. PBB concentrations were consistently highest in liver, followed by kidney, brain, and plasma levels. Geller et al. (*89*) used operant conditioning techniques to evaluate whether known doses of PBBs affected the learning or performance of a discrimination task by laboratory rats. Neither the learning nor the performance of this task was affected by administration of doses up to 6 mg/kg/day for 20 days of dosing. However, extra responses (hyperactivity) occurred in the low dose group (1 mg/kg) whereas decreased responses (CNS depression) occurred in the high dose group (6 mg/kg). This latter observation led these authors to speculate that PBBs may produce behavioral changes similar to those of barbiturate-type drugs which can produce a continuum of excitation and depression depending upon the dose administered. Such speculation needs to be followed by further studies.

Immunotoxicity of PBBs

Following the Michigan accident, a large number of studies reported PBB-associated immunological effects in cattle and laboratory animals. Early studies reported that feeding low levels of Firemaster BP-6 to chickens and guinea pigs caused atrophy of various lymphoid organs and depressed antibody responses in the guinea pigs. Cattle inadvertently exposed to PBBs (accumulating up to 30 ppm PBB in body fat) showed no obvious immune alterations (*91*). Feeding Firemaster BP-6 to dogs for 61 days resulted in lymphoid depletion of lymph nodes, particularly in the T-cell zones, and a reduction in IgG containing lymphocytes in popliteal lymph nodes, but only at near toxic doses. In rhesus monkeys, alterations in B- and T-cell functions occurred after feeding PBBs at concentrations of 1.5 ppm for 5 months and 25 ppm for 10 weeks (93).

Preliminary studies in rodents exposed to PBBs for 30 days at dose rates ranging from 0.03 to 30.0 mg/kg body weight per day showed decreased immune responsiveness at 3.0 and particularly 30.0 mg/kg/day (*94*). In chronic studies (0.1 to 10.0 mg/kg body weight per day, 5 days per week for 6 months) several immunological responses were depressed in mice and rats given the higher dose rates although there was no significant decrease to bacterial challenge (*95*). The authors concluded that although PBBs could induce immune alterations in rodents, its immunological effects were apparent only at exposure levels near doses that caused other significant harm such as liver lesions and decreased weight gains.

Wilson et al. (*96*) reported depressed antibody responses following PBB exposure in mice (167 ppm in the diet for 3 weeks). After 6 weeks of feeding, however, the antibody response returned to normal indicating immunological recovery. Further studies showed the mean survival time of mice fed a diet containing 167 ppm PBB for 3 weeks was not affected by *Plasmodium berghei* infection (*97*). Fraker and Aust (*98*) reported that antibody responses to sheep red blood cells in mice fed diets containing 1, 10 or 100 ppm PBB for 30 days were 80%, 30% or 12%, respectively, of control values, although cell mediated immunity was not affected by this exposure regimen.

In summary, while PBB exposure in most laboratory animals causes immunosuppression, doses that come near to inducing clinical signs of overt toxicity are required.

Reproductive Toxicity of PBBs

Adverse reproductive effects were first observed in PBB contaminated cattle and have been subsequently reported to occur in a number of species. Effects on the reproductive process and on the offspring of three laboratory (rat, mouse, and monkey) and two commercial (cow and mink) mammals will be discussed.

Beaudoin (*99*, *100*) reported that Firemaster BP-6 was fetotoxic and teratogenic in rats. By daily exposure to 5 (and 25) mg/kg/body weight of Firemaster BP-6 for 14 days (starting with the day sperm was identified in the vagina as day 0), resulted in a decreased number of implantation sites and 25 mg/kg gave an increase in resorptions and fetal death (*99*). Doses of 50 and 100 mg/kg produced both effects. Administration of single doses of 400 and 800 mg/kg reduced fetal weight at term and resulted in an increased incidence of cleft palate and diaphragmatic hernia (*100*). Corbett et al. (*101*) also observed an increase in fetal death and resorption and reduced fetal weight at term in pregnant rats exposed to 100 and 1000 ppm Firemaster BP-6, but no increase in the incidence of malformations at these concentrations. No fetotoxic or teratogenic effects were observed in rats given daily doses of up to 40 mg/kg Firemaster FF-1 from days 7-15 of gestation (*102*).

Fetal death, resorption, and decreased fetal weight at term were also observed in mice receiving [illegible]00 ppm of PBB in the feed on days 4-16 of gestation (*103*). Malformations (exencephaly, cleft palate) were observed at concentrations up to 1000 ppm of BP-6 (*104*). No fetotoxicity or teratogenicity was observed when mice were exposed to the major component of Firemaster BP-6, 2,4,5,2′,4′,5′-hexabromobiphenyl at a dose of 40 mg/kg on days 10-16 of gestation (*105*).

Rhesus monkeys receiving dietary levels of 0.3 ppm of Firemaster FF-1 for 7 months prior to breeding had prolonged menstrual cycles, long periods of implantation bleeding, and decreased progesterone levels (*93*). Infants born to these monkeys were small and gained less weight during the first 16 weeks after birth.

Jackson and Halbert (*106*) first reported that cows exposed to Firemaster FF-1 contaminated feed had a 50% reduction in milk production within one month of exposure probably due to reduced food intake. After the feed was changed their appetite improved, but milk production stabilized at only 60% of normal. Cows that were bred 4-6 weeks before the replacement of feed resumed estrus and had to be rebred in the next 2 months, suggesting early embryonic death and resorption. Cows exposed in the last trimester often had difficult deliveries and delivered larger calves usually 2-4 weeks late. Often these calves were dead or died soon after birth. Metritis occurred in all lactating cows and their milk production was low. Twenty pregnant cows began eating less several weeks prepartum and some died before giving birth. Four of these cows had signs of excess amniotic fluid which resulted in one death and two stillbirths. Mercer et al. (*107*) compared the health status of 16 herds exposed to low levels of PBBs with 15 control herds for the years 1972-1974. Milk production, mastitis and percentage of the herd culled because of sterility and death of the calves did not differ between the PBB-contaminated and control herds. Another study (*108*) compared 46 cows from six exposed herds with 40 cows from five nonexposed herds. There were no differences between the numbers of cows yielding calves, calves born alive or abortions and stillbirths. No postnatal deaths or malformations were seen for either group. With regard to the maternal cows, there were no differences in milk production or in incidence of mastitis, milk fever, or metritis. Ferris (*109*) suggested that calf mortality varied between 9.7 and 34.9% in Michigan in 1973, and that differences in mortality could not be attributed to PBB exposure; however, the relationship between calf mortality and PBB exposure needs further investigation. A study (*85*) of pregnant cows fed daily doses (corresponding to 0.0375, 37.5 mg/kg body weight) of Firemaster BP-6 for a total of 60 days, showed no effect on milk production, incidence of mastitis, or number of inseminations required for conception. There were no differences in birth weights of the calves or weight gain by 42, 180 and 202 days, respectively, without seeing toxic effects. However, the pregnant cow had difficult labor and yielded one stillbirth, which was normal on necropsy. The administration of a total dose of 2,650 mg/kg over a 32-60 day period to six pregnant cows resulted in three abortions and three dead fetuses (*85*).

Mink fed 1, 2.5, 6.25 or 15.6 ppm of Firemaster FF-1, or 1.5 ppm-contaminated poultry or 12.0 ppm-contaminated beef for up to 10 months (*110*), exhibited increased mortality. The number of young born alive decreased by 25% with the 1 and 2.5 ppm doses, and by 42% with the contaminated poultry. At these doses, kit weights at birth were also reduced, and the percent mortality from birth to 4 weeks was increased. The PBB-contaminated beef and poultry was more toxic than the Firemaster FF-1 contaminated mixture.

Mutagenicity of PBBs

Hexabromobiphenyl (isomeric composition unspecified) was assayed for mutagenicity in bacterial test systems of *Salmonella typhimurium* (strain TA 98, TA 100, TA 1535, and TA 1537), both with and without metabolic activation by liver preparations from rats and hamsters. The hexabromobiphenyl was not found to be mutagenic (Zeiger, 1980, personal communication).

Firemaster FF-1, when given to pregnant rats in corn oil in six repeated doses of 100 mg/kg body weight, did not produce chromosome aberrations in bone marrow cells (*111*). Similarly, the incidence of chromosome breaks in bone marrow cells of male mice did not significantly increase when the animals received single doses of Firemaster FF-1 in dimethyl sulfoxide (50 or 500 mg/kg body weight). However, the number of chromosome gaps did increase after 24 and 48 hours. The genetic significance of gaps (regions in the chromatid structure that cannot be stained by the Feulgen method) is not clear (*112*). Male rats which were given Firemaster BP-6 in food in a concentration of 5, 50 or 500 mg/kg for five weeks showed no increase in the number of chromosome breaks either in bone marrow cells or in spermatogonial cells (*113*).

In both studies (*111*, *112*) some animals were also injected with colchicine (5 mg/kg body weight) and mitotic and metaphase indices in bone marrow cells were determined. In the study with pregnant rats (*102*), PBBs interacted synergistically with colchicine: bone marrow cells of animals treated with both colchicine and PBBs showed higher metaphase and mitotic indices than untreated animals or animals treated singly either by PBBs or by colchicine. No colchicine synergism was observed in the study with male mice (*112*).

Carcinogenicity of PBBs

Several studies have shown that the liver is one of the target organs for polybrominated biphenyls (*1*, *114*). Some liver lesions produced are persistent and have the potential to develop into tumors as indicated by Kimbrough et al. (*115*). In this study, Sherman strain COBS rats given a single dose of 1000 mg Firemaster FF-1 by stomach tube in peanut oil developed liver lesions described as "hyperplastic" or "neoplastic" nodules. The neoplastic nodule is a manifestation of the cancer causing process in the liver. It is induced by a variety of carcinogenic agents but not by noncarcinogenic chemicals (*116*). The neoplastic nodules occurred more frequently in females although the levels of PBB in the liver were higher in the male rats. Similar atypical liver nodules are observed in rats after six months when the animals were exposed to multiple doses of PBBs (*76*). The study by Kimbrough et al. (*115*) was evaluated by a working group of the International Agency for Research on Cancer in Lyon, France (*5*), and it was found inadequate for the assessment of carcinogenicity. The number of animals (20 of each sex) was considered too small, and the duration of observations (14 months) too short.

In 1980 (*117*) Kimbrough and her collaborators completed three additional studies. The lot of Firemaster FF-1 and the method of administration was the same as that used in the 1978 study. In one group of 65 female Sherman strain COBS rats, 2-month-old animals were given a single dose of 1000 mg/kg body weight, and in a group of 30 females each animal obtained 12 repeated doses of 100 mg/kg body weight, one dose every 3 weeks. After 26 months, the incidence of liver cell cancer (hepatocellular carcinoma) was 41.4% in the single-dose group, and 64.3% in the multiple-dose group. No liver cancers were observed in the corresponding control groups. A third group of 16 female rats 4 months old was given a single dose of 200 mg/kg also by stomach tube. The incidence of neoplastic nodules 18-22 months later was 31.2%, but no liver cancers were observed.

Similar findings have been observed recently in studies on the carcinogencity of Firemaster FF-1 conducted within the National Toxicology Program (draft NTP Technical Report).* Fischer 344/N (CDF) rats of both sexes were given 125 doses (by mouth) of Firemaster FF-1 over a 6-month period (0.1, 0.3, 1.0, and 10.0 mg/kg/day, 5 days per week). After 6 months of treatment with Firemaster FF-1, some animals of each group were kept for observation during their lifetime. Twenty-three months after the end of exposure, different types of liver tumors were noticed in all male rats which received doses at a rate higher than 0.1 mg/kg/day and in all female rats at a dose rate above 1.0 mg/kg/day. Under the conditions of this bioassay, polybrominated biphenyl mixture (Firemaster FF-1) was considered to be carcinogenic for both Fischer 344 rats and $B_6C_3F_1$ mice of both sexes, inducing neoplastic nodules, hepatocellular carcinomas and cholangiocarcinomas in rats and hepatocellular carcinomas in mice.

In interpreting these data, it should be kept in mind that Firemaster FF-1 is a mixture of PBBs containing mainly compounds with six and seven bromine atoms. In addition it contains penta- and hexabrominated naphthalene impurities in concentrations of about 100 ppm and 2% by weight of an anticaking agent, calcium polysilicate. Furthermore, different strains of rats were used in the Kimbrough et al. studies (*117*) and in the Gupta et al. study (*118*). Lots of Firemaster FF-1 were different as well.

Conclusions

Although the hexa-(2,4,5,2′,4′,5′-) and hepta-(2,3,4,5,2′,4′,5′-) bromobiphenyl compounds constitute the major components of Firemaster FF-1 (BP-6), other minor components and impurities have also been detected, which may have their own toxic properties.

PBBs have contaminated the soil and water in Michigan, but there appears to be little uptake of PBBs by plants from soil. Degradation by sunlight although observable under laboratory conditions is not considered a likely mechanism for PBB degradation, nor is microbial action very effective.

Studies in various animal species indicate that PBBs are rapidly absorbed from the gastrointestinal tract and are widely distributed throughout the tissues with major concentration occurring in liver and adipose tissue. In general, PBBs are resistant to metabolism and are slowly and incompletely eliminated from the body, via the bile into the feces. Thus, elimination of larger doses will not be completed in the lifetime of most animals except in those instances where egg-laying and milk production facilitate elimination by these routes. Some metabolism of PBBs can occur and hydroxylated derivatives have been identified in the urine of dogs and pigs and in pig feces. PBBs are potent mixed inducers of liver mixed-function oxidase enzymes, and thus provide a high potential for modifying the toxicity of other chemicals and drugs.

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*Comments and questions about the DRAFT National Toxicology Program Technical Report on the Toxicology and Carcinogenesis Bioassay of Polybrominated Biphenyl Mixture (Firemaster FF-1) should be directed to Dr. B. Gupta, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709 (919-541-3233).

In acute and chronic feeding studies, the initial signs of general PBB toxicity include weight loss and increased liver size, which may be accompanied by a broad spectrum of other biological effects depending upon the species studied. Studies on specific target organs suggest that the effects on the nervous system such as neuromuscular weakness and behavioral depression may be the result of nonspecific toxicity. Immunosuppression has also been observed in most laboratory animals exposed to PBBs, but the doses required are close to those inducing clinical signs of overt intoxication.

Adverse reproductive effects were observed in cattle following the original accident and have been observed subsequently in rats, mice, monkeys, cows and mink as well as other species. In general, very early in pregnancy PBB intake can lead to resorption of the fetuses, while administration of PBBs later in pregnancy can give rise to offsprings with lower birth weight and some malformations. PBBs can readily enter the fetus by crossing the placenta and can also be transferred to newborn via breast milk.

Mutagenicity studies on PBBs to date have been negative but inadequate. Exposure to high doses of PBBs has been shown to cause liver cancer in rats and mice.

The authors thank Mrs. Judith H. Edmonds for typing the manuscript and Dr. Renate D. Kimbrough and her collaborators (Centers for Disease Control) for making available a preprint of their paper. The Toxicology Information Response Center at the Oak Ridge National Library provided excellent literature search support, which was requested and funded by the Information Response to Chemical Concerns Project.

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Environmental Health Perspectives
Vol. 44, pp. 189-196, 1982

Cytotoxicity and Ciliostasis in Tracheal Explants Exposed to Cadmium Salts

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Cadmium salts were examined for their biological effects on ciliated respiratory epithelium in hamster tracheal explants. Cadmium chloride and cadmium acetate both caused significant decreases in ciliary motion when tested at 100 μM and above. Reductions in relative ciliary activity were dose-dependent and were first demonstrable at 8-32 hr. The decreased ciliary motion was accompanied by decreases in two key metabolic compounds (ATP and dehydrogenase) which are normally associated with cell viability. Histopathological examination of cadmium-treated tissues showed an epithelium thinner than normal, with extensive vacuolization and few, if any, intact ciliated cells. The various biological effects exerted by cadmium are presented, along with potential mechanisms of pathogenesis for the observed ciliostasis and cytonecrosis. Decreases in adenosine triphosphate appear to play a critical role in the development of cadmium-related effects on cellular function and metabolism.

Introduction

Cadmium is a toxic heavy metal which humans encounter in air, food, water and soil (*1*). Respiratory intake varies and depends primarily on proximity to smelters and factories (*2, 3*). Such sources can emit up to 15 $\mu g/m^3$. In addition, cigarette smokers inhale approximately 2-4 μg/pack (*1*). The effects of cadmium on respiratory tissue include pulmonary edema (*4*), inflammation (*5*) and cytonecrosis of type I alveolar cells (*6*). Levels of lung cell enzymes (e.g., glucose-6-phosphate dehydrogenase) are also increased (*4*).

A brief exposure to a cadmium aerosol can depress clearance of subsequently inhaled pathogens, accompanied with an increase in mortality (*7*). Since cadmium induces ciliostasis in the trachea (*8*), this increased susceptibility to infection could be related to depressed mucociliary clearance. The ciliostatic effect has been studied in detail with tracheal explant techniques. Concentrations of cadmium chloride as low as 1.4 μg/ml caused a significant decrease in ciliary frequency after a 24-hr exposure (*8*), while another study using cadmium acetate reported such changes in as little as 4 hr (*9*).

The current project was designed to examine more closely the *in vitro* effect of cadmium on the ciliated respiratory epithelium. Emphasis was placed on the correlation of ciliostasis and cytotoxicity, the quantitation of cadmium within target tissue, and the relative effects of cadmium chloride and cadmium acetate.

Methods

Tracheal ring explant cultures were prepared from adult male golden hamsters (*Mesocricetus auratus*) with the techniques previously described by Gabridge et al. (*10, 11*). Basically, the explants consisted of transverse slices of trachea, 1–2 mm thick, the inner surfaces of which were lined with a ciliated epithelium. Cultures were maintained in a thin layer of complete minimal essential medium (MEM; Eagles) with 10% fetal calf serum.

Relative ciliary activity, RCA (*10, 12*), was calculated during observation of the ciliary motion with 225× phase optics on an inverted microscope. RCA is the product of the percent of the surface

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with an intact epithelium (0–100%), and a subjective estimate of vigor of ciliary motion (0–3 +). Biochemical assays of cell viability were based on dehydrogenase activity (primarily succinate, as determined with a tetrazolium chloride reduction assay) (*13*) and ATP content (determined photometrically with a luciferin-luciferase enzyme system) (*14*). For histopathological examination, tracheal explants were rinsed in phosphate buffered saline (PBS, pH 7.4) and fixed in 4% glutaraldehyde in PBS. Specimens were embedded in plastic, sectioned with a glass knife and stained with hematoxylin and eosin.

Solutions of cadmium chloride and cadmium acetate (Mallinckrodt) were prepared in MEM and were filter sterilized with 0.22 μm (mean of diameter pore size) Millipore filter. To determine actual tissue concentrations of cadmium in tracheal explants, neutron activation analysis was employed. Tracheas were rinsed, air dried and stored under vacuum. Samples were heat-sealed in a precleaned polyethylene vial. Known volumes of solution were sealed into similar vials and the sample and standards were sealed in a larger precleaned vial. Vials were irradiated in the rotary sample holder of the University of Illinois Advanced TRIGA Reactor for 8 hr at a flux of 4 × 10^{12} neutrons/cm^2-sec. After a delay of approximately 7 days, the samples were counted for 2 hr for gamma radiation by using a 10% efficient Ge (Li) spectrometer having a 2.1 keV resolution at the 1332 keV line of ^{60}Co. The activities of the sample and standard, counting times and the standard cadmium concentration were entered into the program of the Nuclear Data 6620 computer to calculate the amount of cadmium in each sample. One standard deviation errors (from unknown uncertainties in sample and standard preparation and from the counting statistics) were propagated to calculate the reported uncertainty in the sample vials.

Results

Initial studies were conducted in order to determine the effect of cadmium chloride on tracheal explant ciliary motion. The data (Fig. 1) show that cadmium has very definite ciliostatic activity. Doses of 100 to 750 μ*M* caused significant decreases in the percent and relative vigor of the ciliated epithelium, and the effect was dose dependent (Table 1).

At 100 μ*M*, ciliary motion was nearly normal for the first 8 hr, but then gradually decreased for the next 40 hr. After a total of 48 hr in 100 μ*M* cadmium chloride, ciliary activity was reduced 20%. The drop was much more pronounced in a 250 μ*M* solution. It caused a significant decrease within the first 4 hr. By 48 hr, the 250 μ*M* treatment had reduced ciliary activity to approximately 4% of the control levels. The response to 500 and 750 μ*M* cadmium chloride was even more rapid. A 96% drop in ciliary activity was evident after 24 hr, and by 30 hr the activity was eliminated.

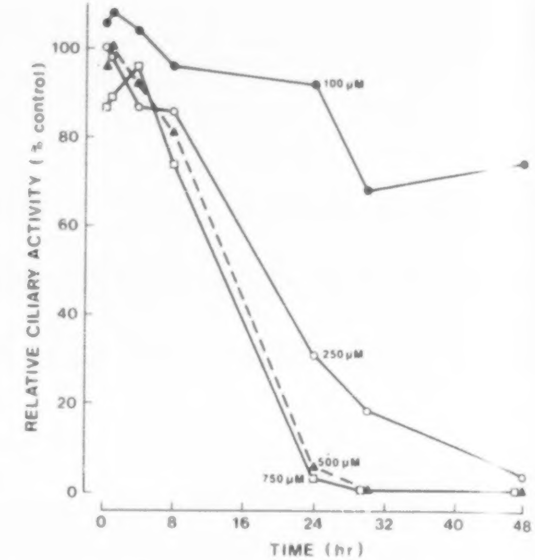

FIGURE 1. Effect of various concentrations of cadmium chloride on ciliary activity in hamster tracheal explants. Mean data from six explants per time point.

Table 1. Effect of cadmium chloride on relative ciliary activity (max = 300) and ATP content of tracheal explants after 24 hr exposure (mean values from six explants).

Concentration, μ*M*	Ciliary activity		ATP content	
	0 hr	24 hr	μg/mg	% of control
0	271	253	1.71	100
100	255	183	1.60	94
250	248	116	0.96	56
500	254	10	0.58	34
750	242	4	0.43	25

These decreases in ciliary motion were correlated with similar decreases in metabolic activity in cadmium-treated explants. Tracheal explants were incubated in 0, 100, 250, 500 or 750 μ*M* cadmium chloride for 48 hr, scored visually for ciliary motion, and then evaluated for dehydrogenase activity. The data (Fig. 2) show that the percent decrease (relative to MEM control explants) in both ciliary activity and enzyme activity are highly correlated. With the 100 μ*M* concentration, both parameters are between 40 and 70%, while with 250 μ*M* and

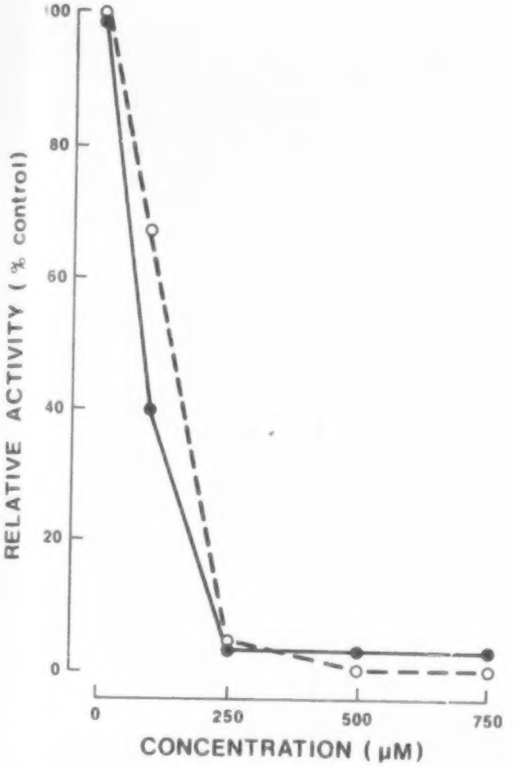

FIGURE 2. Effect of cadmium chloride on (○) relative ciliary activity and (•) dehydrogenase activity of tracheal rings after 48 hr of exposure. Mean data from six explants per time point.

above, the activities range from 0 to 5% after the 48 hr exposure.

A similar, though not as direct, correlation was seen with the ATP content of treated explants. After 24 hr of incubation, the amount of ATP in the tissues was inversely correlated with cadmium dose. Doses of 250 μ*M* caused 50-60% decreases in both ciliary activity and ATP content. Levels of 500 and 750 μ*M* reacted similarly in that they caused reductions in ciliary motion of ≥ 90% and reductions in ATP of ≥ 66%. The fact that both the visual measure of ciliary motion and the biochemical assessments of cell viability decreased in dose-dependent fashions, indicates that cadmium chloride induced both ciliostasis and overt cytotoxicity.

In order to determine whether or not these effects were limited to only the chloride salt of cadmium, cadmium acetate was also evaluated in the tracheal explant system. The results (Table 2) show that the effects of cadmium acetate are similar to those of cadmium chloride. A level of 100 μ*M* cadmium acetate caused decreases of 33-43% in ciliary motion and ATP content after 24 hr. The response to 250 μ*M* was even more pronounced, and also included both the function and metabolism of ciliated cells.

The damage induced by cadmium salts (i.e., ciliostasis and cytonecrosis) was also apparent in histological sections of treated tissues (Fig. 3). The lumen of the control tracheal explants was lined with a pseudostratified layer of ciliated epithelial cells. Cilia were common. The ciliated cells and occasional goblet cells composed the layer above the basal layer of undifferentiated cells. When explants were incubated in 250 or 750 μ*M* solutions of cadmium chloride for 24 hr, significant cytonecrosis could be observed.

The ciliated epithelium was largely destroyed by cadmium. Ciliated cells were fewer in number and the epithelium was thin and sparsely populated. Few ciliated tufts on epithelial cells could be detected. Cells tended to round up and lose their tall, columnar type of shape. Vacuolization was common, both around and within the nucleii. Morphological alterations which suggested massive cell destruction were evident at both cadmium concentrations, with almost complete destruction of the epithelium obvious at 750 μ*M*.

In an attempt to quantitate the amount of cadmium actually present in treated tissues, a neutron-activation technique was used. This method was advantageous, in that it requires small quantities of tissue (< 15 mg) and has high accuracy and sensitivity. Our results (Table 3) indicate that levels in excess of 8,000 μg/g were attained in

Table 2. Effect of cadmium acetate on relative ciliary activity and ATP content of tracheal explants (mean data from six explants).

Concentration, μ*M*	Ciliary activity				ATP content	
	0 hr	3 hr	24 hr	% of control[a]	μg/mg	% of control[a]
0	243	238	203	100	2.26	100
100	222	189	115	57	1.52	67
250	239	228	67	33	1.09	48

[a] At 24 hr.

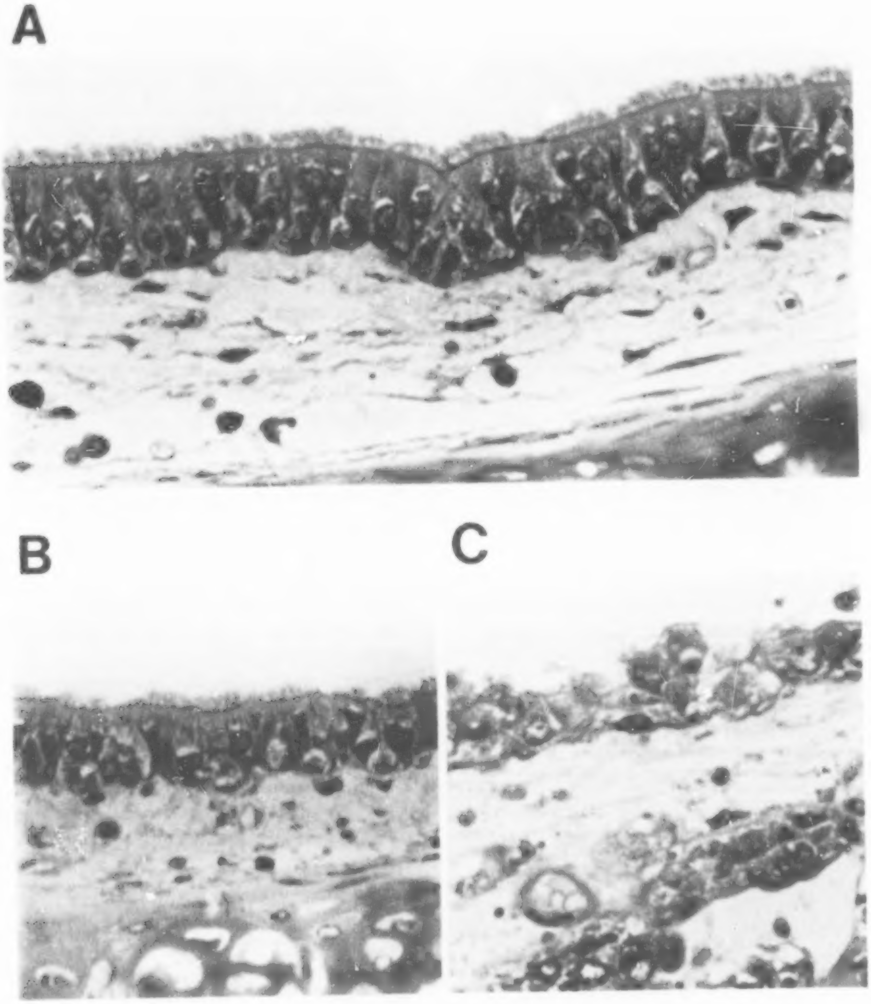

FIGURE 3. Light photomicrographs of control and cadmium-treated hamster tracheal explants after 24 hr: (*A*) MEM control;) 250 μ*M* $CdCl_2$; (*C*) 750 μ*M* $CdCl_2$. Hemotoxylin and eosin; original magnification 600×.

explants after 24 hr in 750 μ*M* solutions of cadmium chloride. Entry of Cd was time dependent. After 1 hr of incubation, only 785 μg/g could be detected (relative to a base-line level of approximately 30 μg/g). The level increased more than 10-fold during the next 23 hr. These data clearly indicate that elemental cadmium is present in tracheal tissues bathed in cadmium salts.

Discussion

Cadmium is an element which is released into our environment in massive quantities. In a recent review (*11*), Fishbein estimated that the arly worldwide release of Cd was approximate 5-8 million lb. Of these emissions, 90% come from etal plating and alloy and battery manufacturing Lev- els of airborne cadmium particulates in the wor lace can be quite high. Values from 110 to 2125 g/m^3 were reported by Lauwerys et al. (*15*). For co par- ison purposes, the levels of cadmium chloride our current study (100 to 750 μ*M*) ranged from 0. 3 to 0.171 g/l.

The *in vivo* effects of cadmium are man and varied. The precise biological response depen s on the route of administration, but most organ dis-

Tab 3. Time dependence for entry of cadmium (750 μM Cd( ) into tracheal tissue (neutron activation analysis of 40 explants per time sample.

T ne, hr	Weight, mg (dry)	Cd content (± SD), μg/g
0	14.33	< 39
1	14.02	785 ± 27
24	13.56	8,560 ± 200

pla some degree of cytotoxicity (*4-8, 16-20*). Lung infl mmation and necrosis are typically seen in res onse to aerosols containing 10 mg/m³, causing a dis iption in mucociliary function. This effect has bee assessed directly in measurements of ciliary mo on (*8, 9*) and indirectly as increased susceptibility o lung infections (*7*).

T e significance of these doses and their conseque ices for the respiratory tract can best be app eciated in a graphic representation of reported air evels of Cd and the various biological effects (Fi . 4). A threshold limit of 200 μg/m³ was set for ind strial workers by the American Conference of Governmental and Industrial Hygienists (*8*). The values in normal air have been reported from several countries, and range from 0.001 to 3 μg/m³. Factory air levels are much higher, however, and range from 5 to 250 μg/m³. Levels in this range have been shown to cause ciliostasis, increased infections and inflammation in laboratory animals (*5, 7, 8*). Levels above 1000 μg/m³ can cause edema and lung cell destruction (*4, 7*). It is apparent that many workers are being exposed to potentially cytotoxic cadmium levels (*15*).

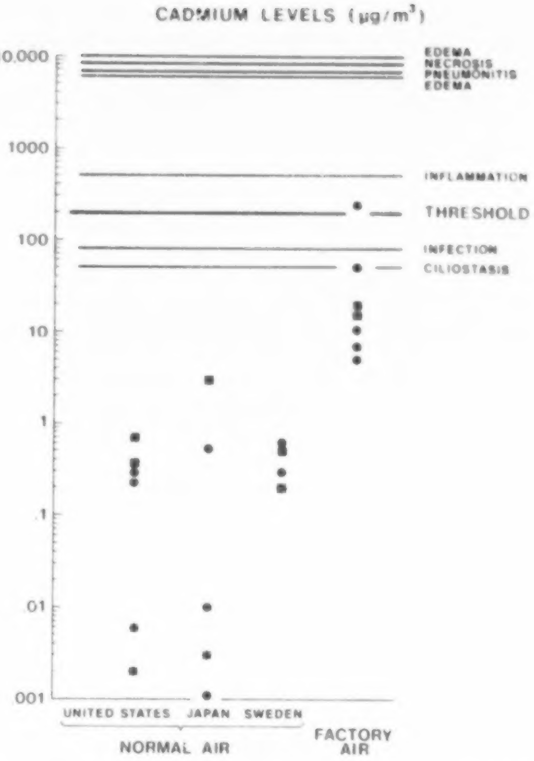

FIGURE 4. Comparison of reported (*1, 3-7*) cadmium levels in air and the amounts required to elicit a biological effect. Squares and circles for each country represent different studies.

The nature of this cytotoxic response in the ciliated respiratory epithelium was the subject of the current study. We chose to use an *in vitvo* analysis because of the ability to control dosage lev ls accurately and to monitor biochemical effects. Oth rs have studied the effects of heavy metals in cell ultures of alveolar macrophages (*21*) and lung fibr blasts (*22*), and in explant cultures of rodent tra iea (*8, 9*). The latter are an especially appropri e model because the target cells are highly diff rentiated and are representative of the mucosal urfaces which would be exposed to inhaled cad iium particles. Unfortunately, the data which we available only pertained to ciliary activity, and t could not be ascertained whether the decreased mo on was due to ciliostasis and/or to cytotoxicity. Th current study examined both effects.

T e ciliated tracheal explants which we treated wit cadmium chloride and cadmium acetate had sig ficant decreases in ciliary motion, consistent wit the observations of Adalis et al. (*8*) and Olsen and Jonsen (*9*). Doses of 100 μM decreased ciliary activity approximately 20% after 48 hrs. A level of 250 μM acted must faster. Little ciliary vigor, and a markedly reduced area of epithelium with ciliated cell movement, were detectable after 48 hr. The 500 and 750 μM levels nearly eliminated ciliary activity within 24 hr.

This ciliostasis was not merely a consequence of physical interference with ciliary movement (e.g., ciliary strands blocked by excessive mucous production) (*23*), but was a direct result of cell necrosis. Assays for ATP content and dehydrogenase activity indicated that treated tracheal cells were metabolically inactive. The decreased ciliary motion and absence of cell viability were compatible with the histopathological analysis. The morphology of cadmium-treated explants was severely affected. The epithelium was thinned, ciliated cells had been lost and vacuolization of both nucleii and cytoplasm was obvious. These data (parameters of structure, function and metabolism) show that cadmium salts are both ciliostatic and cytotoxic for ciliated respiratory epithelial cells. Levels as low as 100 μM can induce chemically detectable changes in 24-32 hr. The fact that this decreased metabolic activity was actually a reflection of cell destruction was established with electron microscopy (manuscript in preparation).

The mechanism by which these cadmium-related alterations in structure and function occur is not clearly understood. Several related theories have been advanced. Sanders et al. (*24*) suggested that cadmium may alter cellular permeability after binding to membranes, or may enter the cell and bind to sensitive target sites such as sulfhydryl groups. This latter possibility seems plausible in view of the protective effect found when compound WR2721 was administered to rats before they inhaled cadmium oxide aerosols. WR2721 is a thiophosphate which is rapidly converted intracellularly to a substrate with highly reactive sulfhydryl groups.

On the level of an intact organism, other mechanisms may be operative. Corradino (*25*) showed that cadmium did not cause a generalized toxicity, but instead specifically reduced the level of calcium-binding protein. This caused cytotoxic effects related to decreased vitamin D-mediated intestinal calcium adsorption. Other studies (*26*, *27*) have illustrated that cadmium can inhibit zinc-dependent enzymes. This is apparently due to competition between cadmium and zinc at cofactor sites on certain enzymes. However, this phenomenon may be species specific, and of more significance in single cell systems (*26*) and laboratory animals than in humans (*27*).

Adalis et al. (*8*) have suggested that cadmium may exert its cytopathology through a diminution of usable ATP. ATP is the energy source for ciliary movement. If ATPase is inhibited by a metallic ion such as cadmium, ciliary activity would be adversely affected. This would be reasonable in light of the data of Sugawara (*28*) and Nechay (*29*). Both investigators demonstrated that cadmium levels as low as 10^{-4} M significantly inhibited (Na^+ + K^+) ATPase and (Mg^{2+}) and (Mg^{2+} + Ca^{2+}) ATPase in animal tissues.

A prolonged lack of available ATP would also decrease cellular protein and nucleic acid synthesis and reduce the efficiency of the ion pumps necessary to maintain normal intracellular osmotic and ionic pressures (*14*, *30*). Our study supports this hypothesis, since it is possible that the measurable ATP losses which we found were a result of all death and thinning of the epithelium after the inactivation of ATPase. Regardless of the precise mechanism, it is clear that cadmium salts can induce cell necrosis and a loss of function which: (a) provides the rationale for the decreased mucociliary clearance and increased mortality from respiratory infections noted in cadmium-treated animals, and (b) underscores the importance for minimizing human exposures to high environmental and occupational cadmium levels.

This work was supported in part by grant HL 26880 from the National Heart, Lung, and Blood Institute. The authors express their gratitude to Dr. P. K. Hopke of the University of Illinois Environmental Research Laboratory for conducting the neutron activation analysis, and to C. Dayton for her editorial review.

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Announcement

Second International Workshop on the Application of Tissue Culture in Toxicology

June 2-4, 1982

This workshop is to be held at Hasseludden, Stockholm, Sweden. Details are available from: Dr. Erik Walum University of Stockholm, Unit of Neurochemistry and Neurotoxicology, Enköpingsvägen 126, S-172 46 Sundbyberg, Sweden.

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NIH PUBLICATION
NO. NIH 82-218

PHTHALATE ESTERS

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ENVIRONMENTAL HEALTH PERSPECTIVES

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service–National Institutes of Health
National Institute of Environmental Health Sciences

VOLUME 45, NOVEMBER 1982

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Subscription rates are as follows: Single copies $8.00, foreign $10.00, domestic $45.00 per year, foreign $56.25 per year. The yearly subscription is for 6 regular issues. Please send check or money order, in advance to the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

The Secretary of Health and Human Services has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through August 30, 1984.

ENVIRONMENTAL HEALTH PERSPECTIVES is a publication of the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services. Opinions expressed herein, however, are not necessarily endorsed nor shared by any of the above agencies.

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Volume 45, November 1982

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Conference on Phthalates

June 9-11, 1981
Washington, DC

Sponsored by the
National Toxicology Program
and the
Interagency Regulatory Liaison Group

Environmental Health Perspectives
Vol. 45, p. 1, 1982

Introduction

by William M. Kluwe*

The use of plastics and plastic-containing products is a reality of modern life that has transformed both the home and work environments. In addition to the polymerized molecules that form the matrix of the plastic, however, other chemicals (adjuvants) are commonly incorporated into plastic products to impart specific use or performance characteristics such as combustion resistance, color enhancement or resiliency. *Ortho* esters of phthalic acid, or phthalate esters as they are generally referred to, have been used to impart flexibility to plastics and may comprise as much as 50% by weight of the final product. Such plasticizers are not polymerized into the plastic matrix and may, with time and use, migrate from the plastic and into the external environment (although the plastic products are obviously designed to retain the plasticizer and, thereby, remain flexible). Readers are referred to an earlier, comprehensive report for more information on the use of phthalate ester plasticizers (*1*).

Di(2-ethylhexyl) phthalate (DEHP) is the most commonly used plasticizer for poly(vinyl chloride) products, including syringes, dialysis tubing and other medical devices. DEHP is also used as a dielectric fluid in transformers and as a hydraulic fluid in heavy machinery. An estimated 400 million pounds of DEHP is produced annually in the United States (*2*). Since all plastic products are ultimately disposed of in the environment, human exposure to DEHP and other components of plastics can occur via contact with water and soil (and air, in some instances), as well as through direct contact with plastic products.

The toxic potentials to humans of DEHP and other phthalate esters were generally perceived to be quite low until the recent report (*3*) by the National Toxicology Program (NTP) that chronic ingestion of DEHP via feed (dietary incorporation) caused tumors in both sexes of rats and mice. The controversy and public interest that surrounded these findings, and their impact on regulations to protect public health, prompted the NTP in collaboration with the U.S. Interagency Regulatory Liaison Group (IRLG) and the Chemical Manufacturer's Association (CMA) to convene a conference to discuss the toxicology of phthalate esters and related issues (Conference on Phthalates, June 9-11, 1981, Washington, D.C.). Scientists from several countries, representing both the public and private sectors, attended and participated in the conference. Selected portions of the scientific sessions and related discussion were submitted to *Environmental Health Perspectives* for publication to reach a wider audience than could attend the conference. The organizers and participants from the Conference on Phthalates wish to thank the editors of *Environmental Health Perspectives* for the opportunity to publish these proceedings.

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Environmental Health Perspectives
Vol. 45, pp. 3-10, 1982

Overview of Phthalate Ester Pharmacokinetics in Mammalian Species

by William M. Kluwe*

Phthalic acid esters, or phthalate esters, are generally well absorbed from the gastrointestinal tract following oral administration. Hydrolysis to the corresponding monoester metabolite, with release of an alcoholic substituent, largely occurs prior to intestinal absorption of the longer-chain alkyl derivatives such as di(2-ethylhexyl) phthalate (DEHP). Phthalate esters are widely distributed in the body, with the liver being the major, initial repository organ. Clearance from the body is rapid and there is only a slight cumulative potential. Short-chain dialkyl phthalates, such as dimethyl phthalate, can be excreted in an unchanged form or following complete hydrolysis to phthalic acid. Longer-chain compounds such as DEHP, however, are converted principally to polar derivatives of the monoesters by oxidative metabolism prior to excretion. A marked species difference in DEHP metabolism exists: primates (man, monkey, some rodent species) glucuronidate DEHP at the carboxylate moiety following hydrolysis of a single ester linkage, whereas rats appear to be unable to glucuronidate the monoester metabolite and oxidize the residual alkyl chain instead to various ketone and carboxylate derivatives. The major route of phthalate ester elimination from the body is urinary excretion. Certain phthalate esters are excreted in the bile but undergo enterohepatic circulation.

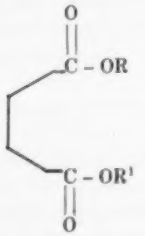
The relationships of phthalate ester pharmacokinetics to their toxicological actions are unknown at the present time, largely due to a lack of elucidated mechanisms of toxic action.

Introduction

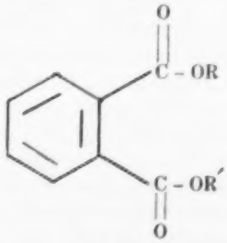
It is the intent of this paper to furnish readers with a general background in phthalate ester pharmacokinetics, with emphasis on absorption, distribution, metabolism and excretion in several mammalian species. Subsequent papers in these proceedings address the absorption and metabolism of specific phthalates in more detail than is contained herein. The greatest amount of data, by far, has been collected on di(2-ethylhexyl) phthalate (DEHP), the most commonly used of the poly(vinyl chloride) (PVC) plasticizers. There seem to be sufficient imilarities in some pharmacokinetic parameters of ie compounds studied to date, however, to permit iferences to be drawn of the pharmacokinetics of ialkyl phthalate esters in general. Attempts to rrelate similarities in pharmacokinetics to a generic attern of toxicity for dialkyl phthalates await ucidation of probable mechanisms of toxic action.

Chemical Structures and Properties

Structurally, phthalate esters consist of paired ester groups on a cyclohexatriene ring (benzenedicarboxylic acid) (Table 1). The *meta* and *para* configurations are known as isophthalates and terephthalates, respectively. The *ortho* configuration, however, is implied in the generic use of the term, "phthalate esters." Phthalate esters are synthesized commercially by condensation of appropriate alcohols with phthalic anhydride as indicated in Table 1. The structures of the ester substituents and the corresponding chemical names of many of



Adipate Esters



Phthalate Esters

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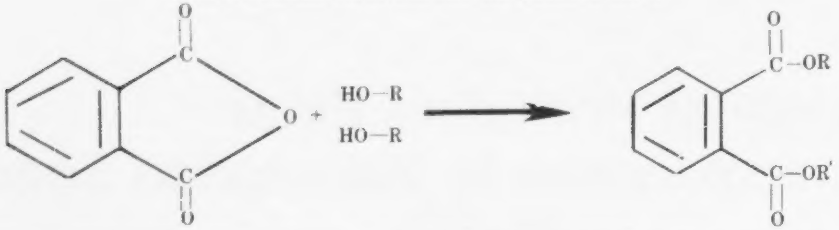
Table 1. Structures of several common phthalate esters.



R	R'	Compound
$-H$	$-H$	Phthalic acid
$-CH_3$	$-CH_3$	Dimethyl phthalate
$-CH_2CH(CH_2)_3CH_3$ CH_2CH_3	$-CH_2CH(CH_2)_3CH_3$ CH_2CH_3	Di(2-ethylhexyl) phthalate
$-CH_2CH_2OCH_3$	$-CH_2CH_2OCH_3$	Di(2-methoxyethyl) phthalate
$-(CH_2)_3CH_3$	$-CH_2C_6H_5$	Butyl benzyl phthalate
$-(CH_2)_3CH_3$	$-CH_2COO(CH_2)_3CH_3$	Butylglycolyl butyl phthalate (Butyl phthalylbutyl glycolate)

the more widely used phthalate esters are also shown. In the related plasticizers, the adipate esters, a four-carbon chain separates the two ester groups.

Specific phthalate esters with short alkyl groups, such as dimethyl and di-*n*-butyl phthalates, are appreciably soluble (e.g., 0.5 g/100 ml) in water (*1*). Most other dialkyl phthalates, however, including DEHP, are relatively insoluble in aqueous mediums because of their lipophilic structures. Volatilities at standard temperature and pressure are generally low, particularly for the long-chain and branched compounds, such as DEHP.

Absorption

Dermal and pulmonary tissues would not be predicted to be major barriers to absorption of phthalate esters from the skin or lungs because of the lipophilic nature of the chemicals. Quantitative (and in most cases qualitative) data on absorption by these routes, however, are nonexistent or unavailable at the present time. Phthalate esters, principally DEHP, can also be introduced directly into the circulatory system by the use of plasticized PVC medical equipment (e.g., syringes or tubing) or by infusions from plasticized PVC blood bags. For other than occupationally or medically exposed populations, however, the most common mode of human contact with phthalate esters is ingestion with food or liquids.

The extent of intestinal absorption of phthalate esters has been estimated by monitoring urinary excretion of the compounds or their metabolites after administering a known amount of compound orally. Shown in Table 2 are the approximate percentages of the ingested dose extracted from the urine of rats given one of several phthalate esters. Absorption of phthalic acid itself appears to be incomplete, but in excess of 90% of oral doses of di-*n*-butyl phthalate over a range of 60 mg/kg to 2.31 g/kg was excreted in the urine within 2 days, indicating complete or near-complete intestinal absorption of this compound. Lesser percentages (~ 40–50%) of DEHP given by gavage in doses of 3 mg/kg or 1.0 g/kg appeared in urine, but greater than 90% of concentrations of 10 or 2000 ppm incorporated in feed were excreted by this route (Table 2). Thus, DEHP in food appears to be very well absorbed from the intestine over a very wide concentration range. The data in Table 2 also indicate that di(3,5-dimethoxyethyl) phthalate, butyl phthalylbutyl glycolate and the DEHP hydrolysis product, mono(2-ethylhexyl) phthalate (MEHP) are all well absorbed from the intestine. Based on this information, it can be speculated that dialkyl phthalate esters, in general, are well absorbed following oral ingestion. In contrast, little is known of the absorptive characteristics of phthalate esters with aromatic alcohol substituents.

Both ester linkages of phthalate esters can be hydrolyzed, leaving phthalic acid as a product. Hydrolysis of a single ester group, however, occurs much more readily than hydrolysis of the second. Esterases capable of generating the monoester metabolite are present in several mammalian tissues (*9*), including intestinal mucosal cells (*10*). Extracellular enzymes present in the intestinal contents are also capable of hydrolyzing DEHP and other phthalate esters (*10, 11*). As shown in Table 3, short-alkyl-chain congeners are more readily metabolized by intestinal contents than are long-alkyl-chain congeners; the extent of completeness of the reaction is also inversely proportional to the concentrations of the diester compounds.

The hydrolytic activity of the intestinal contents

Table 2. Estimation of intestinal absorption of phthalate esters in rats.

Compound[a]	Dose[b]	Time[c]	Percentage of dose excreted in urine	Reference
PA	40 mg/kg	4 days	25	(*2*)
DBP	60 mg/kg	48 hr	90	(*3*)
	270 mg/kg	48 hr	90	(*4*)
	2.31 g/kg	48 hr	90	(*5*)
DEHP	3 mg/kg	4 days	40	(*6*)
	1 g/kg	24 hr	50	(*4*)
	10 ppm[d]	—[e]	> 90	(*4*)
	2000 ppm[d]	—[e]	> 90	(*4*)
MEHP	50 mg/kg	24 hr	70	(*7*)
DiOP	50 mg/kg	4 days	60	(*8*)
BPBG	50 mg/kg	4 days	80	(*8*)

[a] PA, phthalic acid; DBP, di-*n*-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; MEHP, mono (2-ethylhexyl) phthalate; DiOP, di(3,5-dimethylhexyl) phthalate; BPBG, butyl phthalylbutyl glycolate.

[b] Oral gavage, unless specified otherwise.

[c] Period of sample collection, between exposure and termination.

[d] Concentration incorporated into feed.

[e] Time not reported.

Table 3. Hydrolysis of phthalate esters by rat intestinal contents.[a]

Compound[b]	Concentration, mg/ml	Portion metabolized, %[c]	Reference
DMP	1	60	(*11*)
DBP	1	80	
DEHP	1	20	
DOP	1	10	
DEHP	0.1	100	(*10*)

[a] The chemicals were incubated for 16 hr at 37°C under an N_2 atmosphere in 20% (*v/v*) suspensions of gut contents in phosphate-buffered Ringers solution containing 1% (*w/v*) D-glucose.

[b] DMP, dimethyl phthalate; DBP, di-*n*-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DOP, di-*n*-octyl phthalate.

[c] Percent metabolized in 16 hr.

towards phthalate esters *in vitro* is heat (boiling) labile, but unaffected by either filtration of cellular material or by bacteriocidal sterilization of the gut (*10, 11*). Hence, the hydrolytic activity appears to be enzymic in nature, though not of bacterial origin. Albro and Thomas (*12*) have reported very high hydrolytic activity towards DEHP in pancreatic homogenates, implicating the intestinal secretion of pancreatic enzymes as a source of gut hydrolytic activity towards phthalate esters, and predicting that little or no intact diester compound is absorbed from the intestine. The latter hypothesis, that primarily MEHP is absorbed after oral administration of DEHP, raises the question of whether oral and intravenous (and, possibly, inhalation and dermal) routes of DEHP administration represent similar or dissimilar types of exposure.

Distribution

Some of the earlier studies on phthalate ester distributions in the body, particularly those using the intravenous route, were complicated by poor aqueous solubility of the chemicals and artifacts later attributed to phthalate-solubilizer interactions. More recent studies have utilized plasma-solubilized compounds. Virtually all DEHP in blood is protein-bound, approximately 80% to lipoproteins and the rest to albumin (*13*). MEHP in blood, ostensibly a product of DEHP hydrolysis by blood esterases (*14*), equilibrates between free and albumin-bound forms (*13*).

Whether administered by oral or parenteral routes, DEHP and di-*n*-butyl phthalate (DBP), the two compounds studied most extensively, are rapidly cleared from the body (*3-6, 15*). The bulk of the chemicals is cleared within 24 hr and nearly none is left 3-5 days after exposure; there is little or no evidence of tissue accumulation or prolonged tissue retention. The pharmacokinetic behavior of MEHP is similar to that of DEHP (*7*). Fat, absorptive organs (gastrointestinal tract) and excretory organs (liver, kidney, gastrointestinal tract) are the major initial repositories for the dialkyl esters (Table 4). MEHP is not readily deposited in adipose tissue (Table 4), suggesting that perhaps unmetabolized dialkyl compounds, rather than their monoester metabolites, partition most readily into fat. Liver, kidney and gastrointestinal tract probably accumulate the phthalate esters as a mechanism of excre-

Table 4. Distribution of orally administered phthalate esters.

Compound[a]	Species	Dose, mg/kg	Time[b]	Repository organs	Reference
DBP	Rat	60	24 hr	Intestine, adipose, liver, kidney, muscle	(*3*)
	Rat	270	24 hr	Liver, kidney, adipose	(*5*)
			48 hr	None	
DEHP	Rat	500	24 hr	Intestine, stomach, liver, kidney, adipose	(*15*)
	Rat	800	24 hr	Liver, kidney, adipose, muscle, testis	(*4*)
			4 days	Adipose	
DiOP	Rat, dog, pig	50	4 hr	GI, adipose, liver, muscle	(*8*)
			24 hr	GI, adipose	
			4 days	None	
MEHP	Rat	69	24 hr	Intestine, heart, liver, kidney, lungs, muscle	(*7*)
BPBG	Rat, dog, pig	50	4 hr	GI, liver, muscle, adipose	(*8*)
			24 hr	GI	
			4 days	None	

[a] DBP, di-*n*-butyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DiOP, di(3,5-dimethylhexyl) phthalate; MEHP, mono(2-ethylhexyl) phthalate, BPBG, butyl phthalylbutyl glycolate.

[b] Time between administration and examination.

Table 5. Steady-state concentration of DEHP in rat liver and abdominal fat upon prolonged dietary ingestion.[a]

	Dietary concn = 1000 ppm		Dietary concn = 5000 ppm	
Time, weeks	Liver	Fat	Liver	Fat
On diet				
1	50 ppm	4 ppm	120 ppm	25 ppm
2	40 ppm	8 ppm	165 ppm	60 ppm
3	46 ppm	7 ppm	—[b]	—
4	46 ppm	9 ppm	110 ppm	60 ppm
5	36 ppm	8 ppm	115 ppm	80 ppm
Off diet				
1	2 ppm	4 ppm	20 ppm	35 ppm
3	none	3 ppm	none	20 ppm

[a] Data derived from Daniel and Bratt (*6*) with the permission of the authors and Elsevier/North Holland. Calculated from radioactivity recovered in the tissues after dietary administration of ^{14}C-DEHP.

[b] Not determined.

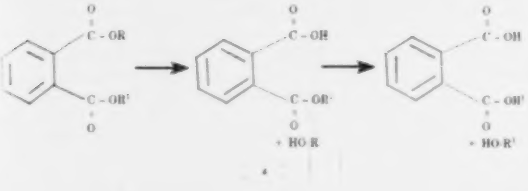
tion (e.g., urine, bile) and may, therefore, be inappropriately labeled as repositories.

Daniel and Bratt (*6*) examined tissue accumulation of DEHP in rats by monitoring radioactivity in fat and liver after dietary exposure to ^{14}C-DEHP. A synopsis of their results is contained in Table 5. Steady-state concentrations in liver were achieved within a week of initiation of treatment, while that in fat required 2 weeks. The organ difference in time to achieve a steady-state level was mirrored by a difference in the rate of decline after removal of DEHP from the diet: radioactivity in the liver was reduced by 80% in 1 week, and was below detection limits within 3 weeks, while radioactivity in fat was reduced only to approximately one-third of the steady-state level within 3 weeks (Table 5). The steady-state tissue concentrations of radioactivity were proportional to the concentration of DEHP in the diet, indicating that saturation of tissue accumulation had not occurred at up to 1000 ppm in the diet. In contrast to high-dose, oral studies in rats, significant fractions of the cumulative dose of DEHP were reported to be retained for several months in the livers of rhesus monkeys infused (intravenously) repeatedly with very small amounts (cumulative doses of 21–69 mg/kg) of DEHP in blood (*16*).

Surprisingly little is known of the abilities of phthalate esters to be transferred to offspring. Radioactivity was recovered from fetal tissues following treatment of the maternal animals with ^{14}C-DEHP or ^{14}C-diethyl phthalate (*17*). The concentrations in the fetal tissues did not exceed those in the maternal tissues. Maternal-fetal transfer of dialkyl phthalate esters across the placenta, therefore, is likely. Excretion into maternal milk is also a distinct probability since lipophilic chemicals readily partition into high fat materials such as breast milk.

Metabolism

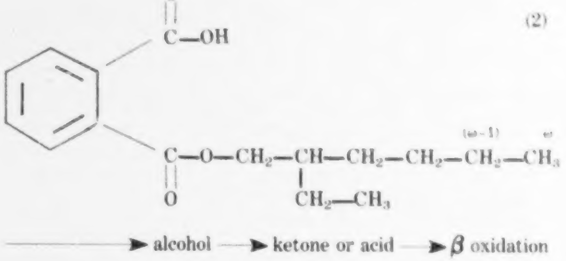
Dialkyl phthalates are metabolized to the monoester products by enzymes present in many tissues, but only those in liver are capable of hydrolyzing DEHP completely to phthalic acid (*12*), as shown in Eq. (1). Appreciable amounts of dimethyl phthalate are excreted as phthalic acid, while only very small fractions of DEHP and other long-chain alkyl phthalates are converted to this product (*18*, *19*).



(1)

Dimethyl phthalate and, to a lesser extent, DBP can be excreted in urine as the parent, unchanged compounds or as their monoester metabolites (*18*). Phthalate esters with longer chain lengths, however, such as DEHP, must undergo further modification after hydrolysis to the monoester to achieve sufficient polarity for renal excretion. The data in Table 6 indicate that several species of animals form glucuronide conjugates with the monoester metabolite of DBP at the free carboxylate group. The ferret, African Green monkey and man form glucuronide conjugates of MEHP, as well, but rats appear to be unable to do so (Table 6). Recent evidence indicates that hamsters, guinea pigs and mice also excrete glucuronide conjugates of MEHP (*20*). The reason for the inability of rats to conjugate MEHP is unknown. In addition to glucuronidation, the residual alkyl chain is oxidized prior to urinary excretion of the metabolites (*3*, *21-26*).

The inability of the rat to metabolize DEHP synthetically requires extensive oxidation of the remaining 2-ethylhexyl moiety to achieve water solubility. A schematic of the metabolism of DEHP in rats, as deduced by Albro et al. (*19*), is shown in Eq. (2). The same general pathways have been documented for metabolism of the monoester derivatives of other dialkyl phthalate esters (*2–7*, *18*).



(2)

Following initial oxidation of the terminal (ω) or adjacent (ω – 1) carbon atom in the side chain to an alcohol, aldehydes, ketones and carboxylic acids are formed via successive oxidations. Compounds with long (six or more linear carbons) alkyl chains may undergo β-oxidation and the loss of two-carbon fragments (*18*, *19*). Generally, the metabolism of phthalate esters is qualitatively unaffected by the route of administration (*6*, *7*, *15*, *18*, *19*).

Excretion

The major route of phthalate ester elimination in both rodent and primate species, including man, is urinary excretion (Table 7) (*23*, *24*). Fecal elimination as a route of excretion has been evaluated for only a limited number of phthalate esters (Table 7). Elimination of di-*n*-butyl phthalate (DBP) in feces is nearly nil over a wide dose range. Appreciable percentages of DEHP appeared in feces when the compound was given by gavage, but whether the fecal material represented unabsorbed DEHP or biliary-excreted material was not ascertained (Table 7). Similarly, only 4 and 9% of dietary DEHP at concentrations of 10 and 2000 ppm, respectively, were recovered from the feces of rats, but it is not known whether these materials were bile-excreted or unabsorbed chemical.

Tanaka et al. (*3*) demonstrated biliary excretion of DBP to the extent of 44% in 24 hr after an oral dose of 60 mg/kg. Only 5% of this same dose was

Table 6. Synthetic metabolism of phthalate esters.

Compound	Species	Route	Dose	Conjugated metabolites	Reference
DEHP	Rat	PO	500 mg/kg	MBP-glucuronide	(*21*)
	Rat	PO	60 mg/kg	MBP-glucuronide derivatives	(*3*)
	Guinea pig	—[a]	—[a]	MBP-glucuronide derivatives	(*3*)
DBP	Hamster	—[a]	—[a]	MBP-glucuronide derivatives	(*3*)
	Rat	Various	Various	None	(*4,6,7,15,18,19*)
	Ferret	PO	600 mg/kg	MEHP glucuronide derivatives	(*22*)
	Monkey	IV	—[a]	MEHP glucuronide derivatives	(*23*)
	Human	IV	94-171 mg	MEHP glucuronide derivatives	(*24*)

[a] Not reported.

Table 7. Biliary, fecal and urinary excretion of DBP or DEHP in rats.

Compound	Dose	Time[a]	Route	Portion of dose, %[b]			Reference
				Bile	Feces	Urine	
DBP	60 mg/kg	24 hr	PO	44	5	88	(*3*)
	500 mg/kg	6 hr	PO	5	—[c]	—	(*21*)
	2.31 g/kg	48 hr	PO	—	5	82	(*5*)
	50 mg/kg	5 hr	IV	10	—	—	(*21*)
DEHP	2.6 mg/kg	4 days	PO	14	56	42	(*6*)
	1.0 g/kg	4 days	PO	—	40 (8)	60	(*5*)
	10 ppm	—[c]	Feed	—	4 (0)	96	(*4*)
	2000 ppm	—	Feed	—	9 (6)	91	(*4*)
	50 mg/kg	7 days	IV	—	28	49	(*15*)

[a] Time of collection post treatment.
[b] Metabolites in parentheses.
[c] Not reported.

eliminated in the feces (88% in urine) of nonbile duct cannulated rats (Table 7), indicating extensive enterohepatic cycling. As only 5% of a much larger dose of DBP, 500 mg/kg, was recovered from bile in 6 hr (Table 7), hepato-biliary excretion of DBP metabolites would appear either to be saturated at high doses or to occur only after a specific period of time post absorption. The latter possibility, delayed biliary excretion, is suggested by the finding that only 10% of an intravenous dose of 50 mg/kg DBP was recovered in bile in 5 hr, in comparison to 44% of an oral dose of 60 mg/kg in 24 hr (Table 7). Biliary metabolites of DBP include monobutyl phthalate, monobutyl phthalate glucuronide and oxidized derivatives of monobutyl phthalate glucuronide (*3*, *5*, *21*).

DEHP can also undergo biliary excretion, as indicated by the report of Daniel and Bratt (*6*) that 14% of an oral dose of 2.6 mg/kg was recovered from bile. Extraction of 28% of an intravenous dose of 50 mg/kg DEHP from feces further indicates that biliary excretion can be a significant route of phthalate ester elimination (Table 7). The biliary metabolites of DEHP in rats were characterized as MEHP derivatives; none were glucuronide conjugates (*6*). Although other studies did not specifically collect bile, an estimate of biliary excretion was obtained by separating fecally eliminated material into parent compound (presumably unabsorbed) and metabolites (presumably bile-excreted products). As only 8% of an oral gavage dose of 1.0 g/kg DEHP was isolated from feces as DEHP metabolites (Table 7), either biliary excretion is not a major route of elimination in this dose range or, more likely, the biliary products are reabsorbed from the intestine and excreted in urine. Similar findings occurred in studies where DEHP was administered with food at concentrations of 10 or 2000 ppm (Table 7). Hence, DEHP metabolites appear to be excreted in bile to an unknown extent, reabsorbed from the intestine, and ultimately eliminated in the urine.

Relationships of Phthalate Ester Pharmacokinetics to Toxic Effects

Speculation on possible relationships between comparative pharmacokinetics and toxicities are tenuous, at best, in the absence of well-defined mechanisms of action. The generally rapid clearance and low potential for tissue accumulation of the dialkyl phthalate esters, however, is at least consistent with, if not the cause of, their low toxic potencies in both acute and chronic studies (*25-27*). The primate/rat difference in ability to form glucuronide conjugates of MEHP is suggestive of a major species difference in chemical metabolism. Whether or not such a difference renders the rat an unsuitable model of human response to DEHP exposure awaits elucidation of the mechanisms of DEHP toxicity in rats. However, other species that differ from rats in their ability to glucuronidate MEHP (e.g., mice) respond with the same types of toxic injury as do rats (*25*), indicating possible independence of chemical toxicity from the ability of the experimental subjects to form glucuronide metabolites. Yet to be determined are the precise mechanisms among several species of oxidation of the residual alkyl group and whether or not potential differences in the nature of the oxidation reaction (e.g., NADH-consuming or NADH-producing) could affect the toxic response. In light of the recent report that DEHP is a hepatocarcinogen in rats and mice (*25*), these species differences or potential differences in DEHP metabolism provide a strong stimulus for studying the mechanisms of mammalian response to phthalate ester exposures.

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Environmental Health Perspectives
Vol. 45, pp. 11-17, 1982

Toxic Potential of the Plasticizer Di(2-ethylhexyl) Phthalate in the Context of Its Disposition and Metabolism in Primates and Man

by Carl C. Peck* and Phillip W. Albro†

Although human toxicity from exposure to the plasticizer di(2-ethylhexyl) phthalate (DEHP) is unknown, reports of animal toxicity from DEHP have stimulated extensive toxicological studies. In the absence of direct toxicity data, information on the disposition and metabolism of DEHP in primates and man may enhance our assessment of the toxic potential of DEHP in man. Studies of DEHP disposition and metabolism in the African Green monkey and man show that the compound is rapidly and extensively metabolized. It is excreted largely in the urine (> 90%) as conjugated (glucuronide) oxidation products of mono(2-ethylhexyl) phthalate; excretion in feces accounts for the other 10% of the administered DEHP. Plasma disappearance of parenterally administered DEHP is equally rapid so that by 24 hr following DEHP administration, plasma DEHP concentrations are virtually undetectable, while greater than 70% of the dose has been excreted in urine and stool. The transience of DEHP in primates and the extent to which it is metabolized and conjugated may play a role in the observed lack of toxicity.

Introduction

Interest in the toxic potential of the principal plasticizer contaminant of blood storage, di(2-ethylhexyl) phthalate (DEHP), was kindled by reports of DEHP residues in tissues of patients receiving stored blood products (*1*). However, discovery of DEHP in tissues of humans who had not received transfusions (*2*), serious disagreement between research laboratories on results of assay determinations of DEHP in identical aliquots of DEHP-spiked tissue samples (*3*), and frequent assay contamination with ubiquitous environmental DEHP (*4*), all have made reports of DEHP detection in human tissues difficult to interpret.

Potential DEHP toxicity has been inferred from numerous animal studies. However, many studies of the toxicity of DEHP in animals have entailed nonintravenous routes of administration, atypical dosage formulations, and excessive doses which do not imitate the exposure pattern in man (*5-7*). Correspondingly, the relevance of such studies to human parenteral exposure at relatively low doses of protein-bound DEHP in stored blood products is not amenable to rigorous scientific scrutiny at present. Two exceptions to this criticism bear mention. Garvin et al. (*8*) reported that no acute or cumulative toxicity could be demonstrated in rats infused twice weekly for 9 weeks with DEHP-rich stored homologous plasma. Each infusion resulted in a DEHP dosage which was equivalent to that which a human adult would receive from a 12-unit whole blood transfusion. On the other hand, Jacobson and co-workers (*9*) attributed hepatotoxicity to DEHP in rhesus monkeys given weekly autologous infusions of DEHP-rich platelet concentrates for 1 year. The DEHP infused each week was roughly equivalent to that a human would receive from a 2-unit whole blood transfusion. Whether these investigators correctly interpreted their results as DEHP toxicity is uncertain, since the exposure levels of mono(2-ethylhexyl) phthalate (MEHP) were not reported (see discussion on the significance of MEHP below). The importance of paying careful attention to the details of comparable dosage, the physical state of the dosing formulation and its composition in the planning and interpretation of DEHP toxicity studies has been emphasized (*10*).

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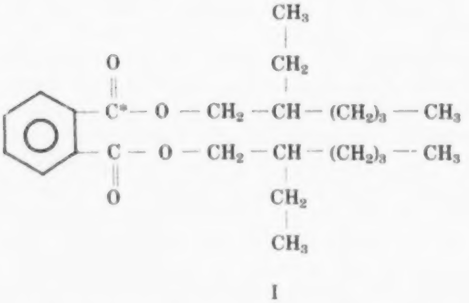
Although human toxicity to DEHP through blood transfusions has not been documented, there remains a concern for the potential toxicity of this ubiquitous compound. In the absence of direct clinical toxicity data, the potential for various effects may be evaluated from knowledge of tissue distribution, the chemical nature of DEHP metabolites and conjugates, the organs involved in DEHP metabolism and elimination, and the time course of these phenomena. It is in this context that we review current knowledge of the disposition and metabolism of DEHP in primates and man.

Structure and Leaching of DEHP

The chemical structure of DEHP is shown as I. DEHP consists of a benzene ring to which are attached two carboxyl groups. Each of the latter is coupled to a 2-ethylhexanol side chain by an ester bond. The asterisk on the carbonyl carbon indicates the position of a 14-C label which was used in our studies of the disposition of DEHP in the primate African Green monkey (*11-13*). Either carbonyl carbon may be the site at which plasma and tissue esterases in primates act to cleave one of the 2-ethylhexanol side chains to produce MEHP.



I

DEHP is a clear, oily liquid which is highly fat-soluble and poorly water-soluble. In poly(vinyl chloride) (PVC) blood storage containers, the plasticizer resides in the PVC matrix as a semisolid and readily migrates from the plastic into plasma during storage. DEHP accumulates in plasma during 4°C liquid whole blood storage at a rate of approximately 1 mg/unit/day and 6 mg/unit of platelet concentrate/day during storage at room temperature (*14*).

Studies performed in our laboratory and else where (*14-16*) indicate that plasma DEHP undergoe hydrolysis to MEHP during storage, presumabl under the influence of plasma nonspecific lipase This reaction results in accumulation of MEHP i whole blood (0.8-2.9 mg/unit after 21 days of stor age), as well as in platelet concentrates (0.8 mg/platelet pack after 50 hr of storage). Interest i MEHP stems from evidence indicating that larg oral doses of MEHP are hepatotoxic in rodents (*17*

African Green Monkey Studies

We initiated our investigation of the dispositio of DEHP in primates by studying the distributio elimination, and metabolism of this compound i the African Green monkey (*11-13*, *18*). In order t

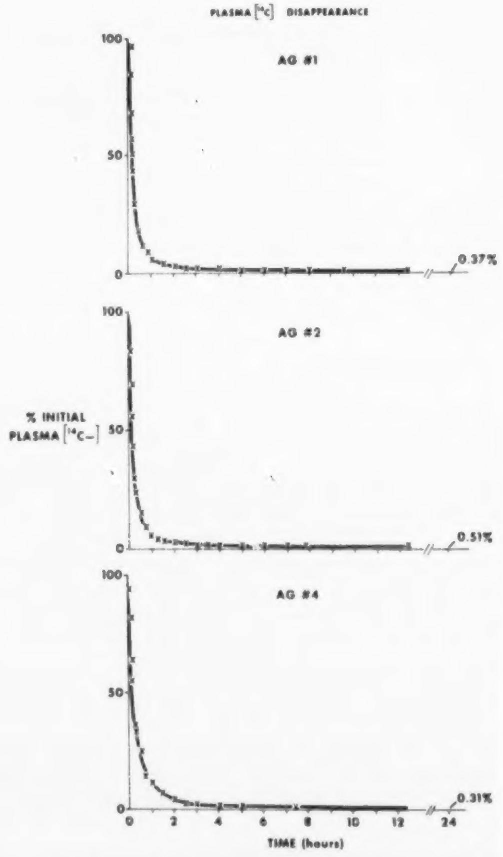


FIGURE 1. Plasma ^{14}C concentrations in three African Green monkeys (AG 1, 2, 4) following bolus injection of ^{14}C-DEHP leached into autogolous plasma. The solid line running through the data (×) was generated from a computer fit of the data (*13*).

mulate closely the manner in which man is exposed DEHP when receiving blood products, we proared a strip of PVC plastic (Fenwal Laboratories, orton Grove, Ill.) which was impregnated with C-DEHP. This sheet exhibited physical characristics which were identical to that of Fenwal's L-146 blood bag plastic. A 20-ml aliquot of plasma as taken from each of three African Green monys; PVC strips containing ^{14}C-DEHP were imersed in these plasmas and stored at 4°C for up to months. At the time of reinfusion, these plasmas ich contained around 3 mg of ^{14}C-DEHP, which as equivalent to that in a two-unit infusion of 1-day-old bank blood in a human. Thin-layer hromatography (TLC) and gas chromatography mass-spectrometry (GC-MS) analysis (*18*) of one of the incubated plasmas revealed the ^{14}C to be distributed as 96% ^{14}C-DEHP and 4% ^{14}C-MEHP: this pattern closely resembles that of unlabeled phthalates in human plasma stored in conventional PL-146 blood bags. Following bolus infusion of the ^{14}C-DEHP-laden autologous plasma, serial plasma, urine and stool samples were obtained and counted for ^{14}C activity.

As can be seen in Figure 1, plasma ^{14}C concentration rapidly declined, so that by 90 min after infusion there was less than 5%, and after 12 hr there was less than 1% of the initial ^{14}C concentration. Characterization of the chemical structures of the ^{14}C compounds in plasma during the first 30 min after infusion, revealed rapid conversion of DEHP to MEHP and more fully oxidized MEHP derivatives (Fig. 2).

Figure 3 shows the buildup of ^{14}C in urine following the infusion. It can be seen that by 4 hr after infusion, greater than 50% of injected ^{14}C had been excreted in the urine, and that by 24 hr, greater than 70% had been excreted by the kidneys.

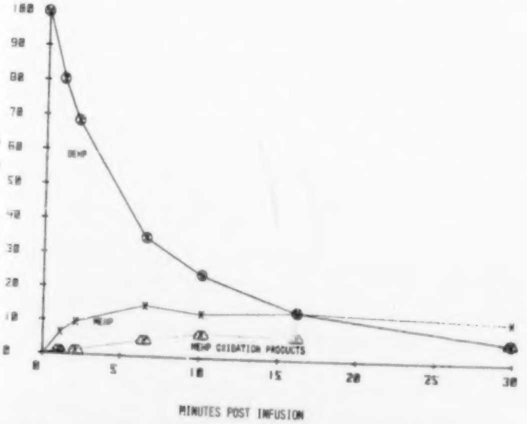


FIGURE 2. Plasma DEHP and metabolites of DEHP during the first 30 min following injection of ^{14}C-DEHP leached into autologous African Green monkey plasma (*13*).

Table 1 reveals the chemical structure of DEHP metabolites in urine of the African Green monkey as identified by GC-MS (*18*). The predominant metabolites were the 5-ethyl, isohexanol monoester of phthalic acid and MEHP. More than 80% of urinary metabolites were conjugated to glucuronide. The cumulative urinary excretion patterns of DEHP metabolites during the first 4 hr are plotted in Figure 4.

Fecal ^{14}C excretion accounted for up to 8% of the injected ^{14}C by 48 hr after infusion (Fig. 5). The theoretical cumulative urinary excretion of ^{14}C at infinite time (beyond 4-5 days) coupled with the measured fecal excretion of ^{14}C accounted for the entire injected dose of ^{14}C-DEHP.

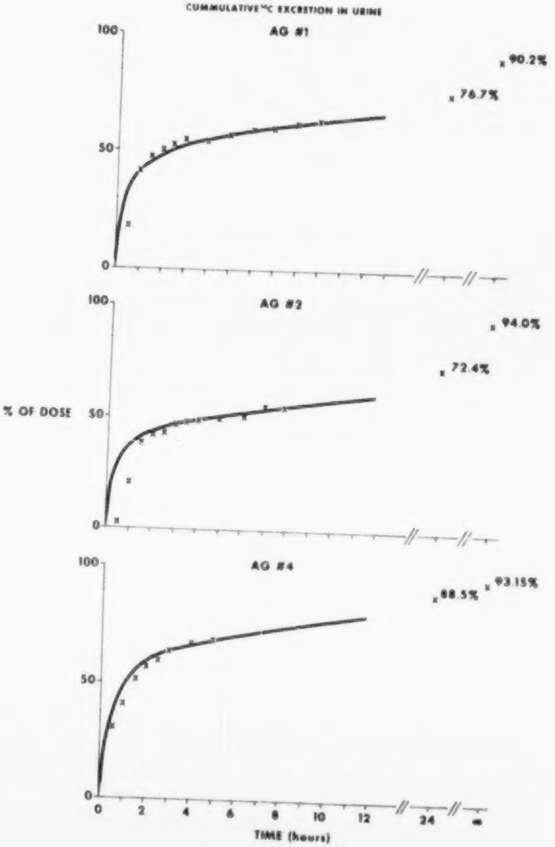


FIGURE 3. Cumulative ^{14}C excretion in urine of three African Green monkeys following bolus injection of ^{14}C-DEHP leached into autologous plasma. The solid line running through the data (×) was generated from a computer fit of the data (*13*).

Table 1. DEHP metabolites in urine of an African Green monkey (AG#4).

Metabolite		
Code	R[a]	% of dose 4 hr after infusion
12 B	CH_2-$CH(C_2H_5)$-$(CH_2)_2$-CHOH-CH_3	26.1
MEHP	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-CH_3	19.6
Unhydrolyzed[b]		5.4
11	CH_2-$CH(CH_2CH_2OH)$-$(CH_2)_3$-CH_3	3.2
10	CH_2-$CH(C_2H_5)$-$(CH_2)_2$-CO-CH_3	3.1
11 X	CH_2-$CH(C_2H_5)$-(CH_2)-CHOH-CH_2-CH_3	2.7
9 E	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-COOH	2.7
DEHP[c]	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-CH_3[c]	<1.8
9 A	CH_2-$CH(C_2H_5)$-(CH_2)-COOH	Trace
9 C	CH_2-$CH(C_2H_5)$-$(CH_2)_2$-COOH	Trace
13 B	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-CH_2OH	Trace
Phthalic acid	H	Trace
Total		66.9

[a] Structure: o-C_6H_4 (COOX)COOR, where X = glucuronide or H.

[b] By β-glucuronidase or sulfatase.

[c] X = R.

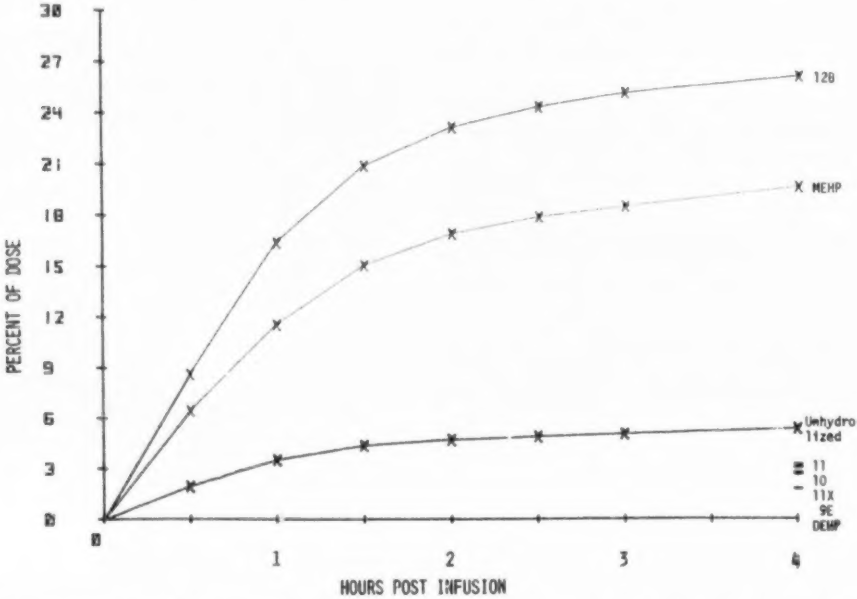


FIGURE 4. Cumulative excretion of individual DEHP metabolites in urine of one African Green monkey (AG 4) following bolus injection of ^{14}C-DEHP in autologous monkey plasma. The metabolite codes are the same as those in Table 1.

DEHP Disposition and Metabolism in Man

A detailed study of the disposition and metabolism of DEHP in two cancer patients receiving larger doses of DEHP via platelet concentrate infusions has been reported (*13*, *19*). One patient (case I) received 94.7 mg of DEHP in 4 hr, and another (case II) received 174.3 mg of DEHP in 1.5 hr. Figure 6 shows the cumulative urinary excretion of all DEHP derivatives. More than 50% of the infused dose appeared as DEHP derivatives in urine within 6 hr. This pattern is similar to that seen in the African Green monkey (cf. Fig. 3).

Table 2 is a list of the chemical structures of the urinary metabolites (case II) which were identified by GC-MS (*18*). As in the African Green monkey,

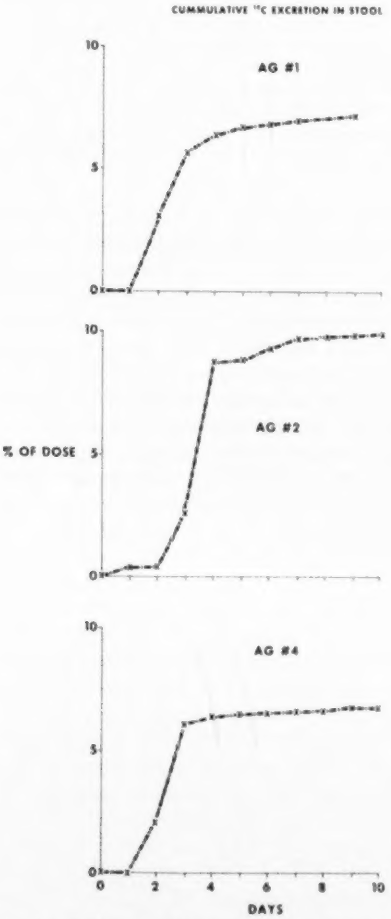
Table 2. DEHP metabolites in human urine (case II).

Metabolite		
de	R[a]	Cumulative % of dose 24 hr after infusion[b]
B	CH_2-$CH(C_2H_5)$-$(CH_2)_2$-$CHOH$-CH_3	23.4
	CH_2-$CH(CH_2CH_2OH)$-$(CH_2)_3$-CH_3	8.3
	CH_2-$CH(C_2H_5)$-$(CH_2)_2$-CO-CH_3	7.7
EHP	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-CH_3	6.9
-X	CH_2-$CH(C_2H_5)$-CH_2-$CHOH$-CH_2-CH_3	6.1
-E	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-$COOH$	4.7
-D	CH_2-$CH(CH_2COOH)$-$(CH_2)_3$-CH_3	1.5
-B	CH_2-$CH(COOH)$-$(CH_2)_3$-CH_3	1.2
-B	CH_2-$CH(C_2H_5)$-$(CH_2)_3$-CH_2OH	Trace
otal		59.8[c]

Structure: o-$C_6H_4(COOX)(COOR)$, where X = glucuronide (80%) or H.

Each is underestimated by < 2% due to sample loss.

Underestimated by ≈ 6.1%.



URE 5. Cumulative excretion of ^{14}C in stool of three African Green monkeys (AG 1, 2, 4) following injection of ^{14}C-DEHP in autologous plasma.

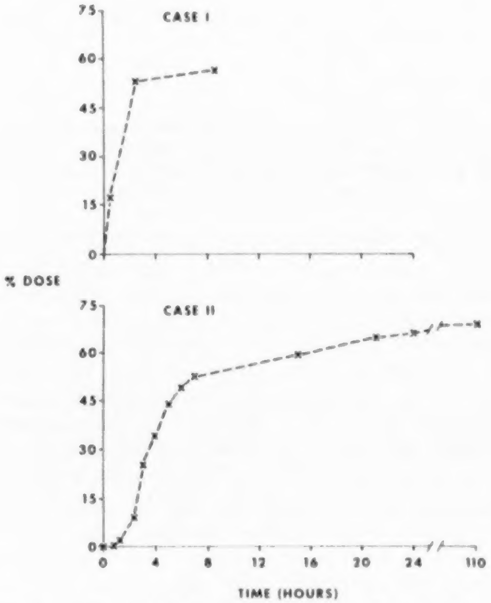


FIGURE 6. Cumulative urinary excretion of total DEHP metabolites in two cancer patients following platelet concentrate infusions containing leached DEHP.

the predominant metabolite was the 5-ethyl, isohexanol monoester of phthalic acid, but in contrast to the urinary metabolite pattern in the monkey, MEHP is fourth on the list and is followed by four more fully oxidized derivatives of MEHP. As in the monkey, approximately 80% of the urinary metabolites were conjugated to glucuronide.

Figure 7 shows the cumulative urinary concentrations of various DEHP derivatives in case II throughout the first 24 hr after infusion.

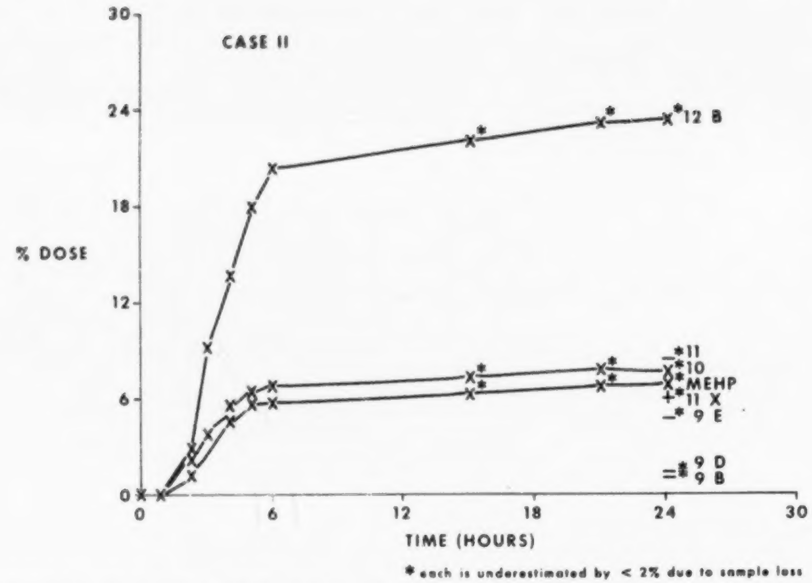


FIGURE 7. Cumulative urinary excretion of individual DEHP metabolites in a cancer patient (case II) following a 1.5-hr infusion of concentrated platelets containing 174.3 mg of leached DEHP. The metabolite codes are the same as those in Table 2. Asterisks (*) denote values underestimated by < 2% due to sample loss.

A study of the disappearance of DEHP in human plasma following infusion of DEHP-laden platelet concentrates (*20*) enabled its pharmacokinetic characterization (*21*). Plasma disappearance half-life was 30 ± 12 min, apparent distribution volume was 2819 ± 383 ml/m^2 and clearance was 78 ± 20 ml/min-m^2 ($n = 6$). Based upon these average pharmacokinetic parameters, Figure 8 shows the plasma DEHP time profile for a simulated platelet infusion in which 90 mg DEHP in 530 ml of pooled platelet concentrate is infused in a 30-min period into a 70-kg man. The DEHP is initially distributed into a volume which is slightly larger than blood volume, rapidly distributes to other tissues and disappears from plasma so that it is essentially undetectable 4 hr after infusion.

Discussion

Consideration of these kinetic and metabolic data in the light of potential toxicity in humans leads to some preliminary answers and invites new questions. The restricted apparent distribution space, rapid disappearance from plasma, rapid urinary excretion and extensive hydrolysis, oxidation, and glucuronide conjugation could all be interpreted as "detoxification" mechanisms. Such an interpretation, however, requires knowledge of tissue sensitivities to DEHP and its metabolites. In the absence of such information, DEHP disposition data contribute to our assessment of potential DEHP toxicity in several ways. For instance, the extensive metabolism and rapid excretion of DEHP metabolites cast serious doubt on the contention that DEHP found in human tissues is a result of blood transfusions or exposure from other environmental sources; contamination during sample preparation and assay appears to be a more likely explanation. Moreover, the rapid excretion of the bulk of DEHP metabolites within 8 hr following infusion of blood products suggests that DEHP-related toxicity is more likely to be acute in onset rather than a late-appearing or long-term effect. Any toxicity observed may be the effect of a metabolic product of DEHP rather than the parent compound. In this regard, the reports of MEHP accumulation during blood product storage (*14, 15*) and description of its hepatotoxicity in rodents (*17*) should focus future research efforts on this compound in particular. Moreover, since the enzymes responsible for hydrolysis, oxidation and conjugation of DEHP and MEHP may be located primarily in the liver, this organ, as well as the kidney, will be exposed to relatively high concentrations of DEHP derivatives. This knowledge should focus DEHP toxicity research on these two organs, as well as alert investigators to

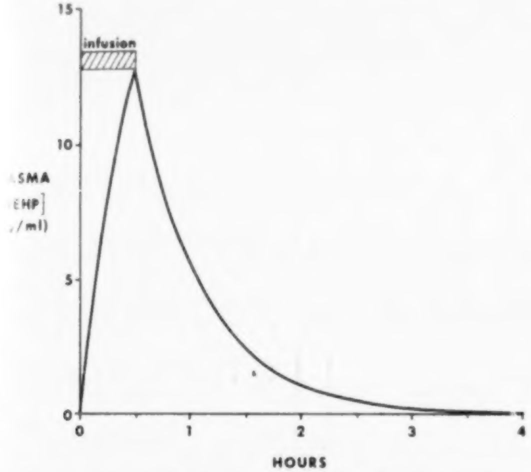


FIGURE 8. Simulated human plasma DEHP concentrations during and after a 30-min infusion of 530 ml of pooled 72-hr-old platelet concentrate (90 mg DEHP total). The simulation is based on average pharmacokinetic parameters derived (*21*) from data reported on six patients receiving DEHP-rich platelet concentrates (*20*), and assumes a body surface are of 1.73 m^2.

the possibility that toxicity may appear with altered DEHP metabolism or excretion due to hepatic or renal disease.

There is no evidence at present that humans exposed to DEHP via DEHP-laden blood products or from other sources experience any adverse effects from DEHP exposure. The studies of DEHP disposition reviewed in this paper demonstrate that DEHP administered parenterally to primates and humans is rapidly converted to conjugated hydrolyzed oxidation products of MEHP which are rapidly excreted in the urine. These metabolic processes account for elimination of more than 50% of the infused DEHP by 8 hr after infusion, while elimination of the entire dose is virtually complete in 4-5 days. Whether these dispositional events account for the lack of observed human toxicity from DEHP remains an unanswered question.

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Environmental Health Perspectives
Vol. 45, pp. 19-25, 1982

Pharmacokinetics, Interactions with Macromolecules and Species Differences in Metabolism of DEHP

by Phillip W. Albro,* Jean T. Corbett,* Joanna L. Schroeder,* Sandra Jordan* and Hazel B. Matthews*

The recent long-term carcinogenesis bioassay of di(2-ethylhexyl) phthalate (DEHP) in rats and mice reported by the National Toxicology Program was the first such bioassay to implicate DEHP as a hepatocarcinogen. At the levels of DEHP fed (up to 1.2% of the diet for two years), the livers of the rats would have been exposed to unhydrolyzed diester; this would not have been the case at lower dosages. Extrapolation to lower dosages is therefore questionable. We do not have sufficient pharmacokinetic data in mice to evaluate the dose relationships as yet. Rodents differ conspicuously from primates in their manner of metabolizing DEHP, both in terms of the demand made on the oxidation potential of the liver and in the chemical properties of the major metabolites. The relevance of these differences must be determined before rodent species can be considered models for the effects of DEHP in humans. Radioactivity from carbonyl-labeled DEHP did not associate with purified protein, RNA or DNA from rat liver *in vivo*. Label from 2-ethyl-(1-^{14}C)-hexyl-labeled DEHP or mono(2-ethylhexyl) phthalate (MEHP) did appear to associate strongly with purified DNA, but label from free ^{14}C-labeled 2-ethylhexanol did not. The apparent binding from DEHP and MEHP was not exchangeable, but was not proven to be covalent. This phenomenon needs additional study.

Introduction

The studies to be presented in this report were undertaken in response to the recent announcement by the National Toxicology Program (NTP) that di(2-ethylhexyl) phthalate (DEHP), fed at high levels in the diet to rats and mice for two years, was a hepatocarcinogen. We wanted to know something about the possible mechanism of carcinogenesis in rodent liver and also to explore the relevance of the extremely high doses used to produce it.

Materials and Methods

Dose Dependence of Distribution/Elimination

The first study relates to the effect of the dosage of DEHP on its metabolism, distribution and excretion. Preliminary experiments with both Sprague-Dawley (CD) and Fischer 344 rats revealed that the maximum amount of DEHP that could be given as a single oral dose without significant excretion of unabsorbed DEHP in the feces was 200 mg/kg body weight. Pharmacokinetic studies using ^{14}C-labeled DEHP at doses above this level would rapidly become dominated by unabsorbed DEHP and could not be directly compared to rates of elimination at lower doses.

We chose to give DEHP by gavage in cottonseed oil rather than incorporated into the solid diet for several reasons. First, we wished to avoid the problem of determining the amount of food actually eaten, and the problem of achieving a comparable dose among replicates on an *ad libitum* schedule. Second, we wished to avoid the possibility of contamination of urine and feces with radioactivity from food crumbs. Most importantly, doctoring feed with high levels of substances that can be smelled alters feeding habits. Feed containing low levels of DEHP is consumed over a shorter time interval than food containing a high level of DEHP. In order to have DEHP dosage be the dominant variable, then, it was necessary to administer the compound by gavage.

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To lessen the discrepancy between the NTP bioassay feeding conditions and the present study, the DEHP was given each day at 5 P.M., shortly before the beginning of the dark cycle. The rats usually began feeding shortly thereafter, so absorption occurred in the presence of the normal dietary nutrients.

The first study involved 36 male Fischer 344 rats, 12 in each of three groups (A, B and C). The rats received NIH 31 chow and water *ad libitum*. Feces and urine were collected daily at 4:30 P.M. and stored frozen. Group A rats received 1.8 mg of [7-^{14}C]-DEHP (carbonyl label) per kilogram body weight per day. Group B received 18 mg/kg per day, and group C received 180 mg/kg per day. All received the same amount of radioactivity, with different amounts of nonradioactive DEHP diluent. Rats received a total of 10 daily doses of DEHP.

Three rats from each group were sacrificed 1, 3, 10 or 12 days after receiving their first dose of DEHP. Various tissues and feces were radioassayed using a tissue oxidizer. Urine was radioassayed directly in liquid scintillation fluid. The profiles of radioactive metabolites in urine were determined by HPLC as described previously (*1*). Lipid-soluble metabolites were extracted from liver (*2*) and chromatographed in Florisil with 15% diethyl ether in hexane. Unhydrolyzed DEHP was measured by radio-TLC of the eluate from Florisil on silica gel GF in hexane:diethyl ether:acetic acid, 120:30:1.5 (v/v).

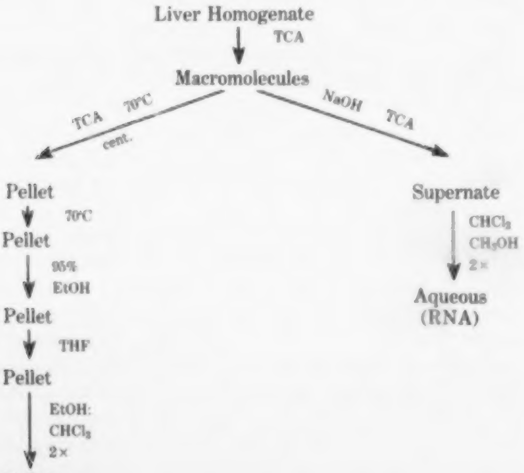


FIGURE 1. Outline of purification procedures for protein and RNA from rat liver. TCA = trichloroacetic acid.

Absorption Thresholds

In a separate experiment rats and mice were, in triplicate, given single oral doses of DEHP in cottonseed oil ranging from 1.8 mg/kg body weight to 1 g/kg. The animals were sacrificed 6 hr later, as this was the time after dosing at which the maximum concentration of ^{14}C was found in the livers. The livers were assayed for intact DEHP as described above. The results of this experiment were compared to the level of DEHP found in the livers of rats fed a diet containing 1% DEHP until the level reached a steady state (≤ 6 days).

Species Dependence

The distributions of DEHP-derived metabolites in urine of Fischer 344 rats, CD mice, Syrian golden hamsters, and Hartley albino guinea pigs were determined following single oral doses of DEHP in cottonseed oil by techniques applied previously to urine from CD rats (*1*, *3*, *4*,), African Green monkeys (*5*) and humans (*6*). In general, the distributions were independent of dose up to the amount of DEHP that caused intact ^{14}C-DEHP to appear in the feces.

Labeling of Macromolecules

In the last type of experiment to be reported here, male Fischer 344 rats were fed diets containing either 0.01% or 1% DEHP, unlabeled, *ad libtum* for 11 days. The rats were given a tracer dose of labeled DEHP or one of its metabolites on day 1,

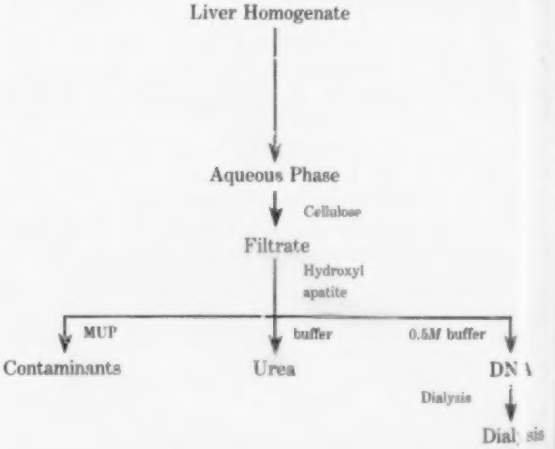


FIGURE 2. Outline of purification procedures for DNA from rat liver. MUP = buffer containing urea. For details see Markov and Ivanov (*7*).

6 or day 10. Rats given labeled DEHP on either 1 or day 10 were sacrificed on day 11; those n labeled DEHP on day 6 were sacrificed on 7 (24 hr later). Livers were homogenized, after ch protein, RNA and DNA (*7*) were isolated in ly purified form. The purification procedures briefly diagrammed in Figures 1 and 2. The tions were tested for cross contamination by ctrophotometric assays (*7-9*), after which they e radioassayed, as were aliquots of the original nogenate, by digestion in NCS and dispersion in uasol.

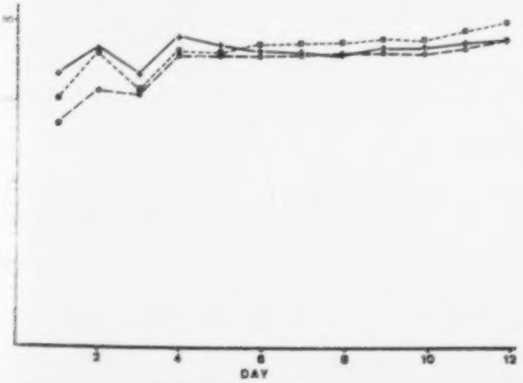
Results and Discussion

Dose Effects

The cumulative excretion of ^{14}C expressed as a percentage of the cumulative dose is diagrammed in Figure 3. After an "adaptation period" of about 4 days, excretion became quite independent of the dose. On an absolute basis, rats in group C were eliminating ^{14}C at a rate 100 times that of the group A rats. Up to a dose rate of 180 mg/kg per day, then, there was no indication of even beginning to saturate the overall elimination mechanism.

Looking more specifically at the liver, we saw (Table 1) that the percentage of the dose of ^{14}C retained in the livers tended to decrease with time and also with increasing dose. The mean rates of dropoff in radioactivity after dosing was stopped were not statistically significantly different in the three groups. There was no evidence, then, for accumulation in this organ.

Essentially the same observations applied to the testes as were noted for liver (Table 2), except that testes never reached as high concentrations of DEHP metabolites as did liver.



URE 3. Cumulative excretion of ^{14}C in urine and feces as a percentage of the cumulative dose for male rats receiving (○) 1.8, (□) 18, or (+) 180 mg of [7-^{14}C]-DEHP per kg body weight per day by gavage in cottonseed oil, for 10 successive days.

Table 1. Percentage of ^{14}C fed left in liver (average of three).

	^{14}C left in liver, %		
Day	1.8 mg/kg/day	18 mg/kg/day	180 mg/kg/day
1	0.64 ± 0.31	0.45 ± 0.16	0.25 ± 0.08
3	0.44 ± 0.27	0.35 ± 0.03	0.15 ± 0.01
10	0.13 ± 0.05	0.15 ± 0.01	0.12 ± 0.02
12	0.06 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
10–12 % hr	0.93 ± 0.27	0.53 ± 0.07	0.62 ± 0.14

Table 2. Percentage of ^{14}C fed left in testes (average of three).[a]

	^{14}C left in testes, % (± SD)		
Day	1.8 mg/kg/day	18 mg/kg/day	180 mg/kg/day
1	0.0142(0.0028)	0.0148(0.0065)	0.0094(0.0049)
3	0.0060(0.0017)	0.0065(0.0005)	0.0064(0.0020)
10	0.0020(0.0004)	0.0019(0.0010)	0.0015(0.0006)
12	0.0037(0.0008)	0.0025(0.0010)	0.0032(0.0005)

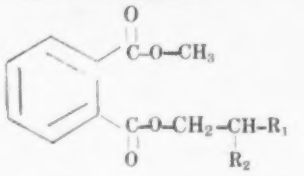
[a] ANOVA; $F_c = 1.0159$, $p = 0.4169$; $F_R = 31.30$, $p = 0.0005$.

The structures of the urinary metabolites of DEHP that still retain aromaticity are shown in Table 3. These compounds are grouped according to functionality, or roughly according to the extent of oxidative metabolism needed to produce them, in Table 4. This table indicates that the only statistically significant effect of the dosage of DEHP on metabolite distribution in rat urine was a slight increase in free phthalic acid with increasing DEHP dosage. This probably reflects a high K_m for mono-(2-ethylhexyl) phthalate (MEHP) of the lipase that produces phthalic acid. A previous study of lipases and esterases in various rat tissues (*10*) indicated that the only detectable MEHP-hydrolase was located in liver microsomes. It is also possible that this enzyme was induced at high levels of DEHP, but there is no direct evidence at the present time.

Absorption Threshold

As the size of a single oral dose of DEHP was increased, a threshold was reached above which there was a steady climb in the amount of unhydrolyzed DEHP reaching the liver of rats. This is diagrammed for Fischer rats in Figure 4. Fischer rats on a diet of NIH 31 chow fortified with DEHP by weight reached a steady-state level of intact DEHP in their livers corresponding to the peak level predicted in Figure 4. That is, by keeping track of the amount of feed eaten per day per kg body weight, and treating the amount of DEHP

Table 3. Structures of DEHP metabolites after treatment with diazomethane.



Metabolite	R_1	R_2
I	$-CH_2-CO_2-CH_3$	$-CH_2-CH_3$
II	$-(CH_2)_3-CH_3$	$-CO_2-CH_3$
III	$-(CH_2)_2-CO_2-CH_3$	$-CH_2-CH_3$
IV	$-(CH_2)_3-CH_3$	$-CH_2-CO_2-CH_3$
V	$-(CH_2)_2-CO_2-CH_3$	$-CH_2-CH_3$
VI	$-(CH_2)_2-CO-CH_3$	$-CH_2-CH_3$
VII	$-(CH_2)_3-CH_3$	$-CH_2-CH_2OH$
VIII[a]	$-CH_2-CHOH-CH_2-CH_3$	$-CH_2-CH_3$
IX	$-(CH_2)_2-CHOH-CH_3$	$-CH_2-CH_3$
X	$-(CH_2)_3-CH_2OH$	$-CH_2-CH_3$
XI[b]	$-(CH_2)_3-CH_3$	$-CH_2-CH_3$

[a] Absent in the rat.

[b] Mono(2-ethylhexyl) phthalate.

thereby ingested as though it were a single dose, the concentration of DEHP in liver predicted in Figure 4 was in excellent agreement with what was found on analysis (123 μg/g vs 121 μg/g found).

Making the approximation that rats consume feed to the extent of 10% of their body weight daily, then intact DEHP will reach their livers whenever its concentration exceeds 0.43% of the diet. Levels above and below the threshold are therefore qualitatively different relative to what chemical compounds reach the liver. However, it must be kept in mind that when the diet contains more than about 0.5% DEHP, rats tend to reduce their food consumption.

In contrast to the results seen in rats, we were unable to detect an absorption threshold in either CD-1 or C3B6F1 mice for doses up to 1 g of DEHP/kg body weight. This may reflect the higher level of DEHP-hydrolase in the intestines of mice than in the intestines of rats (*10*).

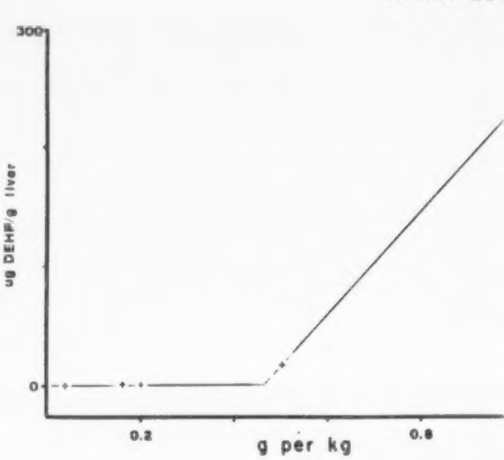


FIGURE 4. Level of intact DEHP in livers of male rats
after a single oral dose of [7-^{14}C]-DEHP in cottonseed o

Association with Macromolecules

Table 5 shows the amounts of radioactivity as ciated with liver homogenates, purified prote RNA and DNA when Fischer 344 rats were give tracer dose of ^{14}C-DEHP or its primary hydroly products. The rats had been eating NIH 31 ch fortified with unlabeled DEHP (1% by weight) fo days to build up the pools of metabolites and int mediates to a steady-state level; the ^{14}C tracer given, and the rats sacrificed 24 hr later. When μCi of carbonyl-^{14}C (7-^{14}C) DEHP was given, detectable radioactivity was found associated w purified macromolecules. However, when the was in the ethylhexyl moiety of DEHP (1′-^{14}C), three classes of macromolecules became labeled the [1′-^{14}C]-DEHP was saponified to phthalic a plus two equivalents of [1′-^{14}C]-2-ethylhexanol bef administration, protein was still labeled, but detectable ^{14}C became associated with DNA. [1′-^{14}C]-MEHP was given, DNA was again label

Table 4. Metabolite distribution as a function of dose of DEHP.

		^{14}C in urine, %		
Dose, mg/kg	Day	-OH side chain	Diacids	MEHP + PA
1.8	1	33.1	66.5	0.4
	3	35.0	64.5	0.5
	10	40.0	58.9	1.1
18	1	38.7	61.0	0.3
	3	38.3	61.3	0.4
	10	36.1	62.8	1.1
180	1	38.1	61.0	0.9
	3	35.5	61.4	3.1
	10	36.1	57.1	6.8

not as efficiently as from [1′-^{14}C]-DEHP. Pro- was much less efficiently labeled from [1′-^{14}C]- HP than from [1′-^{14}C]-DEHP.

the label associated with DNA was covalently nd, which cannot be proven without isolation characterization of the adduct(s), it would appear C-1 (at least) of 2-ethylhexanol is incorporated DNA only when esterified ethylhexanol is given.

any event, it seems clear that the phthalate iety of DEHP is not bound to either DNA, RNA protein in rat liver under these conditions. Chem- analysis of the DNA isolated according to the cedure described detected less than 0.1% RNA less than 0.01% protein.

Table 6 shows the decrease with time in the mount of radioactivity associated with macromole- cules labeled from [1′-^{14}C]-DEHP. The decrease ate for protein was consistent with the average turnover rate of protein in rat liver (*11*) and much slower than would be expected for simple exchange with the unlabeled DEHP from the diet. Labeling of DNA was slightly higher for rats on the 1% DEHP diet than for those on the 0.01% DEHP diet, but there is insufficient information on pool sizes and turnover rates to speculate on this phenomenon. The decrease in labeling of DNA with time was faster for rats on the 0.01% DEHP diet than would be expected if the decrease was simply due to normal DNA turnover. This suggests that some sort of repair mechanism could be involved that was less effective at reducing DNA radioactivity in rats on the 1% DEHP diet. However, much more information would be needed to establish that repair was occurring. At best, this experiment suggests that further studies of the interaction of DEHP with DNA might be worthwhile.

Species Differences in Metabolism

The data in Table 7 are collected from several sources. Urine was collected at intervals after single oral (species other than primates) or IV [African Green monkeys (*5*) and human leukemia patients (*6*)] doses of [7-^{14}C]-DEHP. Urine containing 90% of the total radioactivity excreted was pooled for analysis. Table 7 represents the distributions of radioactive metabolites in pools of urine from three animals except monkeys (two) and humans (two, nonradioactive DEHP). Urine was hydrolyzed with β-glucuronidase for the data in Table 7. A compari-

Table 5. "Bound" radioactivity in macromolecules.

	Liver activity, DPM/g[a]			
	7-^{14}C-DEHP	1′-^{14}C-DEHP	1-^{14}C 2-Et Hexol	1′-^{14}C-MEHP
Homogenate	25918	200247	221600	16460
Protein	0	67805	45180	1331
RNA	0	63000	4350	1878
DNA	0	400	0	114

[a] Measured 24 hr after single oral dose of (a) 100 μCi of carbonyl-labeled (7-^{14}C) DEHP, ethylhexyl-labeled (1′-^{14}C) DEHP, or [1-^{14}C]-2-ethylhexanol + unlabeled phthalic acid, or 50 μCi of ethylhexyl-labeled (1′-^{14}C) mono-ethylhexyl phthalate (MEHP). In all cases the rats had feed containing 1% unlabeled DEHP, and water available *ad libitum*. One gram-equivalent of liver corresponds to approximately 190 mg of protein, 13.5 mg of RNA and 4 mg of DNA.

Table 6. Association of radioactivity with macromolecules.

	Time after [1′-^{14}C] – DEHP given			
	0.01% DEHP diet		1.0% DEHP diet	
	1 day	10 days	1 day	10 days
r activity, DPM/g				
tal	161835	9102	200247	14980
protein	41171	4500	67805	7832
RNA	40040	4402	63000	7471
DNA	310	134	400	293
ein activity, DPM/g				
rotein	230	25	340	42
s RNA	3337	358	5122	607
s DNA	77	33	100	73
crease/10 days, %				
rotein	89.1		88.4	
NA	89.3		88.1	
NA	56.8		26.7	

Table 7. Distribution of phthalate metabolites.

Metabolite	Percentage of total metabolites					
	Rat	Mouse	Guinea pig	Green monkey	Man	Hamster
Residual DEHP	—	0.5	—	2.2	—	0.3
MEHP	Trace	18.6	71.2	28.9	18.3	4.5
I	17.2	16.8	2.4	0.1	—	13.0
II	2.0	1.0	0.4	—	1.8	0.1
III	1.2	0.4	0.5	—	—	0.3
IV	3.3	0.8	0.4	—	1.2	0.4
V	51.3	1.1	6.9	4.2	5.3	14.0
VI	2.6	14.9	1.1	5.9	12.1	10.2
VII	2.6	7.2	0.8	7.0	11.9	4.9
VIII	—	—	—	5.7	8.1	—
IX	13.3	12.3	3.4	38.2	36.2	32.7
X	0.6	2.2	1.3	0.1	0.1	1.9
A, B, C	4.1	1.1	6.2	7.6	4.9	6.1
Phthalic acid	1.8	12.4	5.4	0.1	0.1	9.5

son of extracts from unhydrolyzed and glucuronidase-hydrolyzed portions of urine gave the results shown in Table 8. No conjugates other than glucuronides have been detected in any of the species tested; glycine, taurine and sulfate conjugates have been sought and found absent, in contrast to results reported for substituted benzoic and phenylacetic acid metabolites (*12*).

Most of our investigations of urinary metabolites have involved rats. The metabolite distributions in rats, examined over the past 8 years, have been surprisingly reproducible and independent of factors such as dose of DEHP and age of animal. Rats do not excrete conjugates of DEHP metabolites, an observation that has been confirmed in at least three strains (*5*, *13*). In contrast, each of the other five species examined excretes glucuronide conjugates (Table 6). In most cases, conjugates are the major urinary metabolites.

Whereas primates excrete predominately glucuronides of MEHP, whose formation does not require oxidative metabolism, and metabolites with hydroxyl side chains which require only one round of oxidative metabolism, rats excrete predominately metabolites having carboxyl groups on the side chain (metabolites I-V). These diacids require from three to six oxidative steps for their formation. Carrying oxidative metabolism all the way to the highly water-soluble diacids may be necessary to compensate, in rats, for not making glucuronides.

If formation of hydroxyl side chains involves, by analogy with fatty acid ω-oxidation, a mixed function oxidase reaction as postulated earlier (*3*), one would expect a net conversion of NAD(P)H to NAD(P). The additional steps, from alcohol to aldehyde (or ketone) and from aldehyde to acid, as well as the apparent α- and β-oxidations needed to produce metabolites I-III, would all be associated with net conversion of NAD(P) to NAD(P)H. Thus the overall demand on the oxidation potential of the liver when high doses of DEHP are given would be in opposite directions for rat and primate. To the extent that metabolism of DEHP is involved in its biological activity, then, one must question seriously whether rats can be accepted as a model for man.

Table 8. Forms of excretion products in urine.

Species	Excretion products, %	
	Free	Conjugated
Rat[a]	100	0
Mouse, CD	36	64
Guinea pig	35	65
Green monkey	20	80
Man	20	80
Hamster	85	15

[a]Three strains

Summary

The recent long-term carcinogenesis bioassay in rats and mice as reported at this conference by the NTP is the first such bioassay to report carcinogenesis by DEHP. It is also the first such bioassay to have involved a level of DEHP in the diet high enough to exceed the absorption threshold for unhydrolyzed DEHP. The livers of the rats in the NTP study would have been exposed to moderate levels of intact diester, whereas at levels half those tested or below this would not have occurred. This qualitative difference makes any attempts to extrapolate the hepatocarcinogenesis observed in rats to lower dosage levels questionable. We do not have enough pharmacokinetic data from mice to evaluate the dose relationships as yet.

While we were unable to obtain any evidence for covalent binding of the phthalate moiety of DEHP to macromolecules in rat liver, and there was also no apparent binding of radioactivity from [1′-^{14}C]-2-ethylhexanol to DNA *in vivo*, the labeled carbon atom from either di-([1′-^{14}C]-2-ethylhexyl) phthalate or the corresponding monoester did appear to bind to DNA in rat liver *in vivo*. The diester was a more efficient labeling precursor than the monoester. Disappearance of ^{14}C from the labeled DNA had a time course consistent with the operation of a repair mechanism. If so, repair was less effective when there was a high level of DEHP in the diet. That the apparent binding to DNA was truly covalent has not been proven; however, it was not exchangeable.

We found no saturation of DEHP metabolic pathways in rats over the 100-fold range of dosages between 1.8 mg DEHP/kg body weight per day and 180 mg/kg per day. There was no apparent reason to suspect metabolic saturation in the biological activity of high DEHP dosages. Levels of metabolites in liver and testes fluctuated for about four days on a regimen of daily dosing, then slowly but smoothly declined. This hinted at the operation of an adaptation mechanism, and argued against tissue accumulation.

Rats were unique among six species (including man) tested, in that they did not excrete glucuronide conjugates of DEHP metabolites in urine. Rodent species in general carried oxidative metabolism of DEHP much farther than did primates; the former should experience a net decrease and the latter a net increase in the ratio of NAD(P) to NAD(P)H in liver during exposure to high levels of DEHP. Whereas the major metabolites of DEHP in rat urine are diacids, the major metabolites in urine of primates are glucuronides of hydroxyacids. These species differences in the metabolism of DEHP cast doubts upon the validity of using rats as a model for the effects of DEHP in man.

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Environmental Health Perspectives
Vol. 45, pp. 27-34, 1982

Hepatic Effects of Phthalate Esters

by Prahlad K. Seth*

Di(2-ethylhexyl) phthalate (DEHP), a commonly used plasticizer and microchemical environmental pollutant, produces subtle changes in hepatic function as judged by increase in liver weight and morphological and biochemical alterations. It can modify the biological response of drugs and other xenobiotics. Such interactions appear to occur at the pharmacokinetic phase, as DEHP was found to alter the activity of microsomal drug-metabolizing enzymes and ethanol metabolism. DEHP produced a time- and route-dependent effect on the hepatic cytochrome P-450 contents and activity of aminopyrine *N*-demethylase, aniline hydroxylase, alcohol dehydrogenase and high and low K_m aldehyde dehydrogenases when given orally or intraperitoneally. Under *in vitro* conditions, DEHP produced no effect on the activity of aminopyrine *N*-demethylase or aniline hydroxylase, while mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH) significantly inhibited their activity at concentrations ranging from 2.5 to 15.0 *mM*. Activity of aminopyrine *N*-demethylase and aniline hydroxylase was also inhibited by dimethyl phthalate (DMP) and dibutyl phthalate (DBP) after a single oral administration. In view of the possibility of the human exposure to phthalates and other xenobiotics simultaneously, these observations are of great significance.

Introduction

Phthalic acid esters (PAEs) are commonly used as plasticizers to impart flexibility to plastics, particularly poly(vinyl chloride) (PVC) polymers, which have a wide variety of biomedical and other uses. Di(2-ethylhexyl) phthalate (DEHP) and other PAEs are known to leach out from finished PVC products into blood, physiological fluids, commercial solvents and food materials (*1, 2*). Entry of these plasticizers into the biological system during tranfusions (blood or physiological fluids) or hemodialysis or through food chain and their ubiquitous presence in environment has led to numerous studies on their ology. In spite of their low order of toxicity, P and other phthalates have been shown to hepatotoxic, cytotoxic, teratogenic and muta- effects. Certain renal, pulmonary and repro- ve dysfunctions have also been reported on sure to these plasticizers in aquatic invertebrates mammalian species (*1-3*). Liver and testis ar to be the main target organs in phthalate ity.

is well established that the biological response of xenobiotics can be altered significantly due to their interactions with variables in external and internal environmental and pharmacological agents. Unfortunately, this aspect of phthalate toxicity has not been studied in detail, though such information will be of great significance in assessing the toxicogenic potential of PAEs because of their ubiquity and hepatotoxic nature. This paper therefore reviews some of the hepatic effects of PAEs, with particular emphasis on their interaction with drugs and other xenobiotics.

Effect of Phthalates on Weight, Morphology and Chemical Constituents of Liver

Several investigators have studied the hepatotoxic potential of PAEs in a variety of animal species. Acute and chronic administration of DEHP caused variable effects on liver, depending upon the species of the animal (*4*). In general, phthalates after oral or intraperitoneal administration caused enlargement of the liver (*5-9*), which declined to or below normal weight on prolonged or discontinued exposure (*10*) (Table 1).

Histopathological examination of phthalate-induced enlarged livers showed fatty vacuolation and congestion followed by a cloudy swelling, excessive fatty degeneration (*4, 11*). Dilatation of smooth and rough endoplasmic reticulum, mitochondrial

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Table 1. Effect of DEHP on weight, morphology and chemical constituents of liver.

Species	Route of administration	Principal findings	References
Rat	IP	Liver enlargement; no change in dry weight, moisture, pyruvic acid and lactic acid contents	(*9*)
Rat	Oral	Liver enlargement	(*5,6,8*)
Rat	Oral	Liver enlargement, which disappears on prolonged or discontinued exposure	(*10*)
Rat	Oral	Liver enlargement, proliferation of SER, increase in number of microbodies and swelling of mitochondria with shortening of cristae[a]	(*7*)
Rat	Oral	Cloudy swelling, necrosis and enlargement of liver cells	(*11*)
Mouse	Oral	Swollen mitochondria and increased SER[a]	(*13*)
Ferret	Oral	Increase in liver weight, enlarged parenchymal cells not distributed uniformly, lysosomal changes and dilatation of SER[a]	(*12*)
Monkey	IV	Liver necrosis, inflammatory cell filtration, subtle changes in BSP clearance rate	(*14*)

[a]SER = Smooth endoplasmic reticulum.

Table 2. Effect of phthalates on energy and carbohydrate metabolism.

Species	Route of administration	Principal findings	References
Rat	Oral	Mitochondrial swelling and decreased SDH activity	(*7*)
Rat	Oral	Inhibition of glycolysis and decrease in glycogen content	(*24*)
Rat	IP	Inhibition of SDH, ATPase, diaphorase and cytochrome-c oxidase activities	(*9,15,19*)
Rat	(*In vitro*)	Mitochondrial swelling and stimulation of latent ATPase activity	(*16*)
Rat	(*In vitro*)	Inhibition of mitochondrial respiration, and SDH activity	(*19*)
Rat	(*In vitro*)	Inhibition of mitochondrial respiration	(*17,18*)
Rat	IP	Decreased blood glucose tolerance, glycogen, glycogenesis and glycogenolysis; inhibition of glucose-6-phosphatase, phosphorylase and glucose-6-phosphate dehydrogenase	(*25*)
Mouse	Oral	Decreased glycogen	(*13*)
Ferret	Oral	Absence of glycogen	(*12*)

swelling and increase in microbodies in rat liver have been reported on oral administration of DEHP for 21 days (*7*). Enlargement of cells, dilatation of smooth endoplasmic reticulum and changes in hepatic lysosomes of ferrets (*12*) and swollen mitochondria and increased smooth endoplasmic reticulum in liver of mice (*13*) receiving DEHP orally have also been reported.

Very little information is available on the hepatic effects of phthalates in primates. Jacobson (*14*) has reported liver necrosis and inflammatory cell infiltrations in rhesus monkeys receiving plasma-solubilized DEHP intravenously. Subtle changes in the clearance time of sulfobromophthalein were also observed (Table 1).

Effect of Phthalates on Energy and Carbohydrate Metabolism

Associated with liver enlargement, changes (increases or decreases) in many hepatic enzymes have been reported in phthalate-exposed animals (Table 2). A decrease in succinic dehydrogenase (SDH) activity after oral or intraperitoneal administration of DEHP has been observed consistentl (*7, 15*). Histochemical studies have revealed tha loss of the enzyme occurs specifically in the periporta zones (*7*).

Mitochondria appear to be the target of phthalates Adverse effects of phthalates on mitochondria function have been reported by several investigators *In vitro* studies have shown that several PAEs ca produce significant inhibition of mitochondrial respiration (*16-18*). The studies of Ohyama (*16*) sugges that phthalates are electron and energy transpor inhibitors, while the observations of Inouye et a (*10*) indicate that phthalates can also cause uncouplin of oxidative phosphorylation. Phthalates were foun to produce aberrations in mitochondrial functio e.g., di-*n*-butyl phthalate enhanced K^+ efflux fro isolated mitochondria, usually not produced b standard uncoupling agents (*18*). Sensitivity o various components of electron transport chai towards phthalates was indicated under *in vit* conditions as well as by decreased activities o mitochondrial cytochrome-c oxidase, malic dehydrogenase and diaphorase in liver of DEHP-treate rats (9). The inhibition of these mitochondrial enzymes also indicates an interaction of DEHP with mitochondrial membranes.

DEHP was also found to inhibit the activity of total and Mg^+-stimulated adenosine triphosphatase (ATPase) activity of rat liver (*9*, *19*). Besides liver, the activity of SDH and ATPase was also inhibited in rat heart, lung, kidney (*19*) and gonads (*20*), indicating that suppression of energy-linked reactions may be a generalized effect of DEHP. *In vivo* effects of DEHP (*9*, *15*, *19*) were present 11 days after the final treatment with plasticizers. This suggests that enzymic alterations were perhaps not related to the physical presence of DEHP, as by this time all of the plasticizer would have been excreted (*6*, *21*, *23*).

Detailed studies have been conducted on the effects of DEHP on carbohydrate metabolism (Table 2). A decrease in glycogen in livers of mice (*13*), rats (*19*) and ferrets (*12*) receiving DEHP has been reported. Sakurai et al. (*24*) showed marked depression of glucose and glycogen in livers of rat receiving a diet containing 2% or 4% DEHP. Studies on the rates of incorporation of labeled pyruvate into blood glucose and liver glycogen and alteration in the levels of intermediates of carbohydrate metabolism indicated retardation of gluconeogenesis (*24*). Recent studies of Mushtaq et al. (*25*) using cold and labeled glucose have shown that DEHP inhibits both glycogenesis and glycogenolysis in rat liver. These authors have observed a significant decrease in the activity of glucose 6-phosphate dehydrogenase, phosphorylase and glucose 6-phosphatase and no change in the activity of fructose 1,6-diphosphatase and aldolase in livers of DEHP-exposed rats (*25*).

Effect of Phthalates on Lipid and Protein Metabolism

Since the detection of DEHP in association with triglycerides of beef mitochondria (*26*), its interaction with lipid metabolism has been a subject of active investigation (Table 3). Stein et al. (*27*) showed an interaction between dietary fat and DEHP in lipid metabolism where presence of the phthalate in diet potentiated the growth-promoting effect of lipids in rats. Substantial reduction in serum cholesterol and proliferation of peroxisomes and elevation of catalase and carnitine acetyltransferase activity in livers of animals receiving DEHP via diet was reported by Reddy et al. (*28*). Induction of the fatty acyl-CoA oxidizing enzyme system located in peroxisomes by DEHP has been suggested by Osumi and Hashimoto (*29*), and inhibition of lipid synthesis in rats has been shown by Bell et al. (*30*, *31*). Reduced serum glucose and cholesterol and elevated serum free fatty acid and ketone body have also been reported (*26*, *32*, *33*). The rats receiving 0.5% of DEHP in a normal protein diet showed accumulation of phospholipids, decrease in cholesterol and triglyceride contents in liver and a rise in levels of plasma fatty acids. Such observations suggest the utilization of lipids for the energy source. However, the significance of this altered state of energy metabolism is not understood at present (Table 3).

No significant studies have been conducted on the effects of phthalate on protein metabolism, though their effects on the other proximate principles, carbohydrate and fat have been studied in detail. An increase in the protein content in liver of DEHP-treated rats has been described by Yanagita et al. (*32*). Recently Pillai et al. (*34*) have shown that a DEHP-caused elevation in rat liver protein content is due to a decrease in protein breakdown (Table 3).

Interaction of Phthalates with Other Xenobiotics

Alterations in barbiturate sleeping time on exposure to phthalates have been reported by several investigators. A single intraperitoneal or intravenous injection of DEHP was found to increase the hexobarbital or pentobarbital sleeping time in rats or mice (*6*, *35-37*). Repeated intraperitoneal ad-

Table 3. Effect of DEHP on lipid and protein metabolism.

Species	Route of administration	Principal findings	References
	Oral	Inhibition of lipid biosynthesis	(*30*,*31*)
	IP	Decrease in cholesterol content	(*9*)
	Oral	Decrease in triglycerides and increase in phospholipids	(*24*)
Rat, mouse	Oral	Decrease in serum cholesterol; proliferation of peroxisomes; increase of catalase and carnitine acetyltransferase	(*28*)
Rat, mouse	Oral	Induction of enzymes of fatty acyl-CoA β-oxidation	(*29*)
Rat	Oral	Increase in mitochondrial and microsomal phospholipids; increase in protein content	(*32*,*35*)
Rat	Oral	Increase in protein content (decrease in protein degradation)	(*34*)

ministration of a number of phthalates caused significant and dose-dependent increases in pentobarbital sleeping time in mice (*38*), while repeated oral administration of DEHP resulted in reduced hexobarbital sleeping time in rats (*6*).

DEHP has also been shown to modify the biological response of parathion, an organophosphorus insecticide (*39*). Parathion exerts its toxic effect through its metabolite, paraxon, which is a potent inhibitor of acetylcholine esterase enzyme. A lower inhibitory activity of parathion in DEHP-treated rats suggested an effect of the phthalate on biotransformation of the pesticide to its active metabolite (*39*). Alterations in the biological response of carbon tetrachloride (*40*) and ethanol (*41*) have also been described.

The mechanism by which phthalates modify the biological response of the above-mentioned xenobiotics is not clear, although some investigators have suggested alterations in the biodistribution and biotransformation of the test xenobiotics or an increase in sensitivity of the central nervous system towards them in the presence of phthalates (*6*, *36-39*). Estimation of concentration of barbiturates in blood during sleep and at awakening have shown that sleeping time alterations caused by DEHP were related to retarded disposition of the test drugs and not to the increased sensitivity of the central nervous system (*36*, *38*). An increase in hepatic cytochrome P-450 content, alcohol dehydrogenase activity and persistent inhibition of aniline hydroxylase activity in livers of rats receiving DEHP for prolonged periods indicates its interaction with biotransformation mechanism.

In view of the significance of this aspect of phthalate toxicity and variable effects of phthalates on barbiturate and ethanol-induced sleeping time, the effect of DEHP on some enzymes involved in their biotransformation will be discussed in detail.

Effect of DEHP on Enzymes Involved in Biotransformation of Ethanol and Other Xenobiotics

The effect of DEHP on the activity of aminopyrine N-demethylase or aniline hydroxylase is shown in Figure 1. A single oral or intraperitoneal exposure to DEHP caused a significant dose-dependent decrease in aminopyrine *N*-demethylase and aniline hydroxylase activity and no change in cytochrome P-450 content (data not shown). Exposure to DEHP for 7 days produced a differential effect. Activity of these two microsomal enzymes was significantly increased upon oral administration but remained decreased upon intraperitoneal administration. Repeated oral administration also increased the levels of cytochrome P-450 which were not altered upon single oral administration (data not shown). Cytochrome P-450 levels were also not affected by repeated intraperitoneal administration of DEHP.

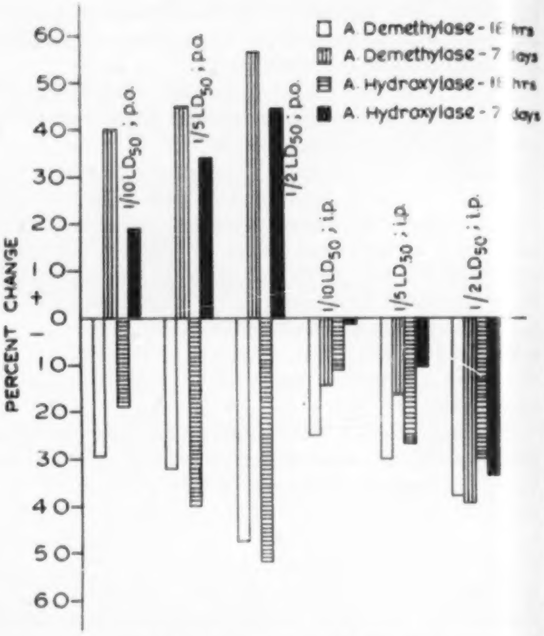

FIGURE 1. Effect of di(2-ethylhexyl) phthalate (DEHP) on aminopyrine *N*-demethylase (A. demethylase) and aniline hydroxylase (A. hydroxylase) activity of rat liver. Adult male albino rats were treated with 1/10, 1/5 and 1/2 of the LD_{50} dose of DEHP orally (LD_{50} = 26 g/kg) by gavage or intraperitoneally (LD_{50} = 50 ml/kg) once or daily up to 7 days. These doses are known to cause teratogenic (*42*), pharmacologic (*38*) and biochemical (*7-9*, *15*, *19*, *20*) effects.

In order to see whether the alterations in activities of aminopyrine *N*-demethylase and aniline hydroxylase are caused by DEHP or its two active metabolites, their effects on these two enzymes were investigated *in vitro* at varying concentrations. DEHP had no effect on aminopyrine *N*-demethylase (Fig. 2) or aniline hydroxylase activity (Fig. 3), while mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH) produced significant inhibition at concentrations ranging from 2.5 to 15*mM*.

The effects of DEHP on activity of alcohol dehydrogenase and high and low K_m aldehyde dehydrogenases are shown in Figures 4 and 5. A single oral administration of DEHP significantly inhibited the activity of alcohol dehydrogenase in a dose-dependent manner while intraperitoneal administration produced the effect only at the highest dose. No effect of DEHP was observed on the activity of high or low K_m aldehyde dehydrogenase after a single oral or intraperitoneal administration (Fig.

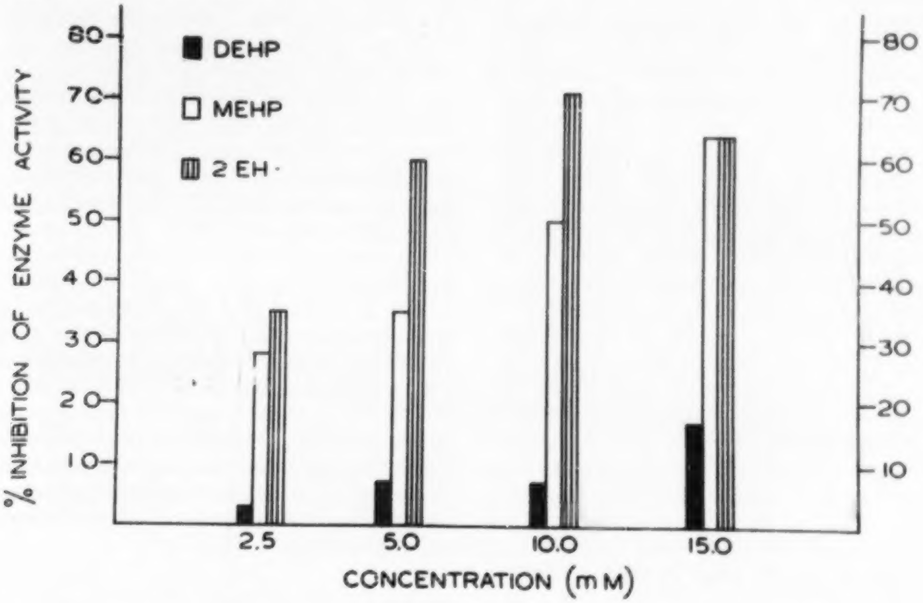

FIGURE 2. *In vitro* effect of di(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH) on the activity of aminopyrine *N*-demethylase of rat liver. These compounds were added directly into the incubation mixture and the effect was studied without any preincubation with enzyme preparation.

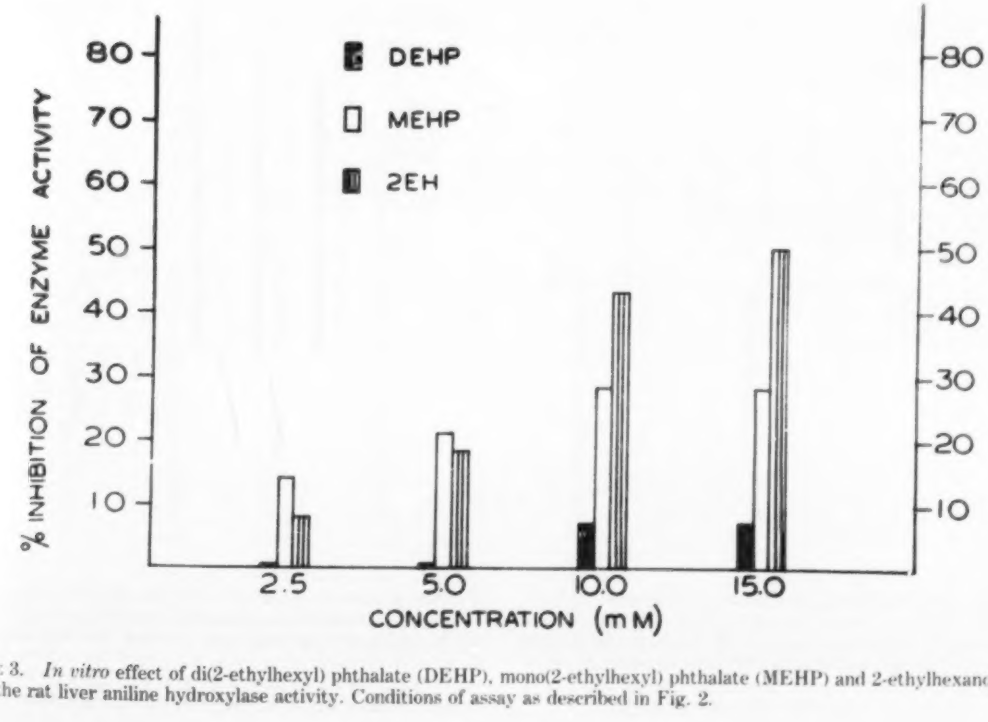

FIGURE 3. *In vitro* effect of di(2-ethylhexyl) phthalate (DEHP), mono(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol (2-EH) on the rat liver aniline hydroxylase activity. Conditions of assay as described in Fig. 2.

4). The repeated oral administration of DEHP produced a different effect than that seen after a single administration of the phthalate. The activities of alcohol and aldehyde dehydrogenases were significantly increased. However, repeated intraperitoneal administration of DEHP caused no significant change in the activity of these enzymes (Fig. 5).

The above results suggest that DEHP produces a time- and route-dependent effect on the activities of aminopyrine *N*-demethylase, aniline hydroxylase, cytochrome P-450 content and alcohol dehydrogenase and high and low K_m aldehyde dehydrogenases. A similar time- and route-dependent effect of DEHP was seen on the pentobarbital (*43*) and ethanol (*44*) sleeping time in rats and mice, respectively. An increase in hexobarbital sleeping time after intraperitoneal or intravenous administration and decrease in the same after repeated oral administration have also been observed by Daniel and Bratt (*6*). However, no suitable explanation has been provided by these investigators. DEHP is hydrolyzed to MEHP at markedly different rates by intestine and liver lipases (*21, 45, 46*) and metabolized to 2-EH. These two major metabolites of DEHP have been reported to mimic some of the biological effects of plasticizers (*7, 16, 33*). Thus, the above observed route-dependent effects of DEHP may perhaps be related to the amounts of MEHP and 2-EH formed in the body, as oral administration of the phthalate may result in higher concentrations of these metabolites.

A significant inhibition of aminopyrine *N*-demethylase and aniline hydroxylase by MEHP and 2-EH without any effect of DEHP on these enzymes under *in vitro* conditions suggests that these two metabolites are responsible for their *in vivo* effects. The DEHP-induced alterations in ethanol metabolism may also be related to MEHP and 2-EH, as these metabolites can be generated both by mitochondria and microsomes (*45*). Such observations are of great significance in view of a recent report on the metabolism of DEHP and accumulation of its metabolites in human blood and red cell concentrates stored in PVC blood bags (*47*).

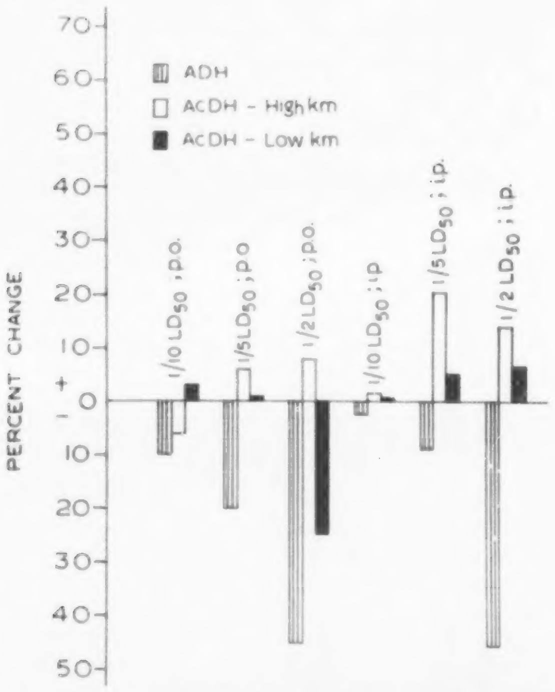

FIGURE 4. Effect of DEHP on alcohol dehydrogenase (ADH), high K_m aldehyde dehydrogenase (ACDH high K_m) and low K_m aldehyde dehydrogenase (ACDH low K_m). Adult male Swiss mice were treated with 1/10, 1/5 and 1/2 LD$_{50}$ dose orally (LD$_{50}$ = 40 ml/kg) by gavage, and enzyme activities were measured in liver 18 hr after the treatment.

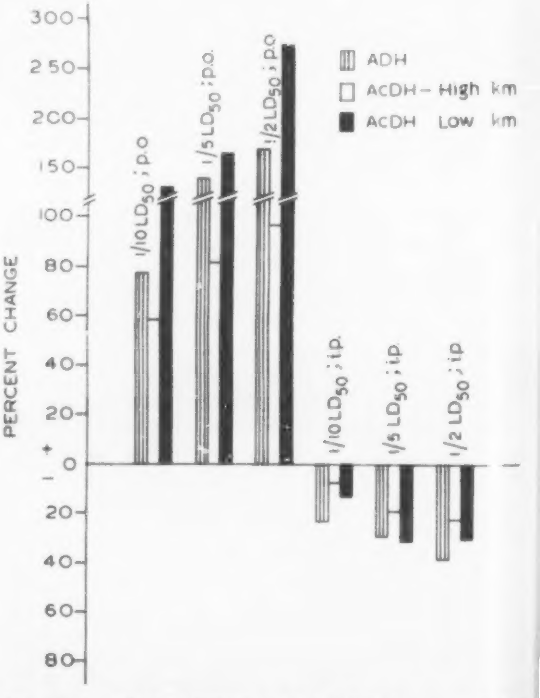

FIGURE 5. Effect of DEHP on alcohol dehydrogenase (ADH), high K_m aldehyde dehydrogenase (ACDH high K_m) and low K_m aldehyde dehydrogenase (ACDH low K_m) activity of mice liver. Adult male Swiss mice were given three different doses of DEHP as described in Fig. 5 daily for 7 days, and enzyme activities were measured 18 hr after the last treatment.

Effect of Dimethyl Phthalate (DMP) and Dibutyl Phthalate (DBP) on Drug-Metabolizing Enzymes

In order to compare the effects of DMP with DBP on the activity of aminopyrine *N*-demethylase and aniline hydroxylase, male rats were exposed to these phthalates at a concentration corresponding to [illegible] of their LD_{50} dose by intraperitoneal route. The activity of these enzymes was examined 18 hr after the administration of phthalates. All the three phthalates inhibited the activity of aminopyrine *N*-demethylase and aniline hydroxylase with DMP producing the maximum effect followed by DEHP and DBP (Fig. 6). The effect of DBP on aminopyrine *N*-demethylase was, however, not significant.

The above results show that besides DEHP, these two phthalates can also inhibit the activity of drug-metabolizing enzymes and hence can modify the biological response of certain xenobiotics. These phthalates failed to exhibit any structure-activity relationship in affecting the activity of aminopyrine *N*-demethylase and aniline hydroxylase. A similar observation on the lack of any role of molecular structure of phthalates in prolonging the pentobarbital sleeping time (*38*) or inhibiting the growth of nerve cell fibroblasts (*48*) has been reported earlier.

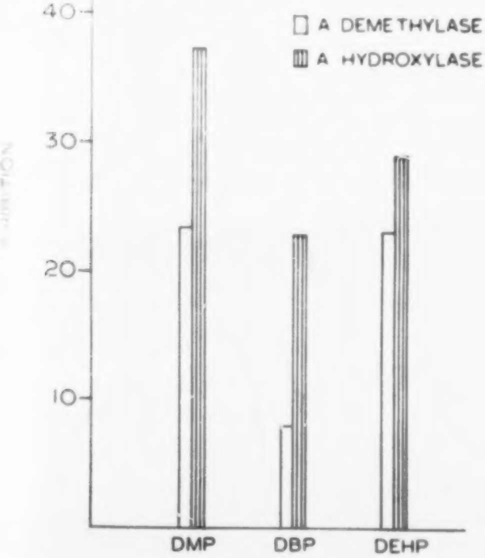

FIGURE 6. Effect of di(2-ethylhexyl) phthalate (DEHP), dimethyl phthalate (DMP) and dibutyl phthalate (DBP) on aminopyrine *N*-demethylase (A. demethylase) and aniline hydroxylase (A. hydroxylase) activity of rat liver. Adult male albino rats were given DEHP, DMP, and DBP (5.0, 3.6 and 3.05 ml/kg, respectively) by intraperitoneal route, and enzyme activities were measured 18 hr after the treatment.

Conclusions

DEHP at high concentrations can cause functional hepatic damage, as reflected by morphological changes, and alterations in the activity of energy-linked enzymes and metabolism of lipids and carbohydrates. Phthalates can interact with other xenobiotics and such interactions occur during the pharmacokinetic phase. These observations are of significance, as man may be exposed to low levels of phthalates for prolonged periods which can cause altered drug-xenobiotic reactions. Effects of DEHP are dose- and route-dependent and are also influenced by strain and species of the test animal. At present, information on the biological effects of phthalates in higher animals is very limited.

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Environmental Health Perspectives
Vol. 45, pp. 35-40, 1982

Phthalate Esters as Peroxisome Proliferator Carcinogens

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The phthalate ester di(2-ethylhexyl) phthalate is both a peroxisome proliferator and a hepatic carcinogen. Peroxisome proliferators as a class are hepatocarcinogenic in rodent species. However, none of the peroxisome proliferators tested to date including the phthalate esters and related alcohol and acid analogs have demonstrated mutagenic or DNA-damaging activity in the *in vitro Salmonella typhimurium*/microsomal or the lymphocyte ^{3}H-thymidine assays. A working hypothesis is proposed that peroxisome proliferation itself initiates neoplastic transformation of hepatic parenchymal cells by increasing intracellular rates of DNA-damaging reactive oxygen production. Evidence which supports such a hypothesis includes increased fatty acid β-oxidation, elevated H_2O_2 levels, accumulation of peroxidized lipofuscin, disproportionately small increase in catalase, and elevated peroxisomal uricase activity which accompany peroxisome proliferation in hepatocytes. Direct testing of this hypothesis will provide insight into mechanisms of phthalate ester carcinogenicity and cytotoxicity.

A xenobiotic which induces the proliferation of peroxisomes in liver cells is designated a peroxisome proliferator. Peroxisomes (microbodies) are single membrane-limited cytoplasmic organelles which functionally have been implicated in gluconeogenesis, lipid metabolism, and the detoxification of H_2O_2. The hypolipidemic agent clofibrate was the first xenobiotic to be identified as a peroxisome proliferator (*1, 2*). Subsequently a large number of other xenobiotics have been discovered to be peroxisome proliferators, including structural analogs of clofibrate (nafenopin, methyl cofenapate, SaH-42,348, and gemfibrozil), hypolipidemic drugs structurally unrelated to clofibrate (tibric acid, Wy-14,643, BR-931), and a group of miscellaneous compounds which were not developed for pharmacological utilization as hypolipidemic drugs but were incidentally discovered to express activity as peroxisome proliferators (*3, 4*). The industrial plasticizer di(2-ethylhexyl) phthalate (DEHP) and related plasticizers, including di(2-ethylhexyl) adipate, are important members of the miscellaneous peroxisome proliferators group (Fig. 1). All peroxisome proliferators studied to date, including DEHP, have been observed to induce hepatomegaly and hypotriglyceridemia when fed to rodents (*5, 6*).

Sustained peroxisome proliferation in rodent liver has been associated with an increased appearance of hepatocellular carcinoma. Peroxisome proliferators which have demonstrated hepatocarcinogenicity in rodents include nafenopin (*7*), Wy-14,643 (*8*), clofibrate (*9*), BR-931 and tibric acid (*10*). The development of liver tumors in animals fed these five structurally diverse hypolipidemic drugs led to the conclusion that peroxisome proliferators as a class are carcinogenic (*10*). Since the formulation of this hypothesis, the industrial plasticizer DEHP has been observed to induce hepatocellular neoplasms in both F-344 rats and B6C3F1 mice (*11*). The reported carcinogenicity of DEHP in rodents is particularly disturbing, since DEHP is a common environmental contaminant (*12, 13*) and it is probable that human exposure to DEHP has been extensive. Our laboratories are currently investigating cellular/molecular mechanisms involved in peroxisome proliferator carcinogenesis, including DEHP and related plasticizers. In this report, possible mechanisms of peroxisome proliferator carcinogenesis based on current knowledge of the properties of these xenobiotics will be reviewed.

Current models of carcinogenesis indicate both an initiation and promotion phase in the formation

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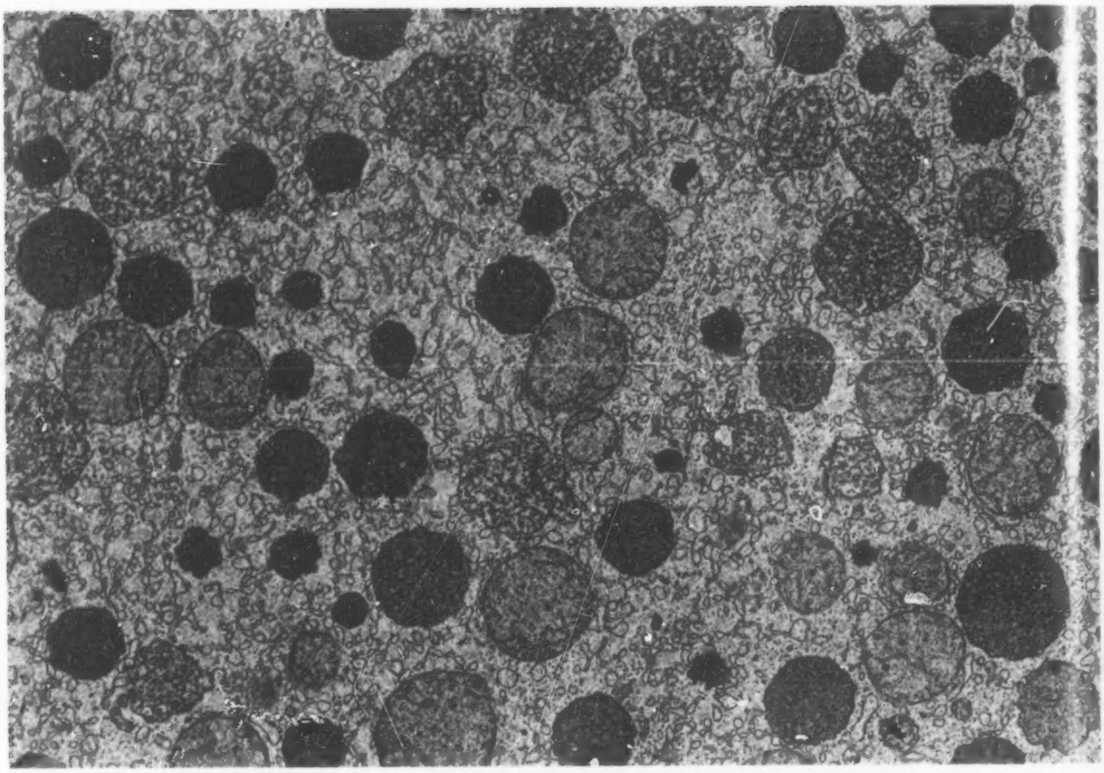

FIGURE 1. Proliferation of peroxisomes induced in F-344 rat liver cell cytoplasm by DEHP. 18,000×.

of neoplasms (*14*). Initiation of cells for neoplastic transformation is irreversible and requires interactions of nucleophilic centers in cellular DNA with electrophilic regions of chemical carcinogens or carcinogen metabolites (*14*). An estimate of the electrophilic reactivity of carcinogens can be obtained by mutagenicity measurements. We have tested five different hypolipidemic drugs (clofibrate, nafenopin, SaH-42-348, Wy-14,643 and BR-931), both structurally related and structurally unrelated to clofibrate, for *in vitro* mutagenic activity against each of the different *Salmonella typhimurium* test strains TA98, TA100, TA1535, TA1537 and TA1538. The hypolipidemic drugs, whether tested without or with rat liver S-9 microsomal preparation, were uniformly negative as mutagens in the Ames *S. typhimurium* assay (*15*). Our results have been extended by de la Iglesia and his co-workers (*16*), who reported that the hypolipidemic drug gemfibrozil or five *in vivo* metabolites of gemfibrozil isolated from rat urine were without mutagenic activity toward the same *S. typhimurium* tester strains utilized by us. Gemfibrozil is both a peroxisome proliferator and hepatocarcinogen in rats (*5*). Also, methyl clofenapate in very recent work was not mutagenic in the Salmonella/microsome assay with tester strains TA98 and TA100 (*17*). We have also utilized the lymphocyte ^{3}H-thymidine assay to test the five hypolipidemic drugs found by us to be negative in the Ames assay plus tibric acid for electrophilic reactivity. The hypolipidemic agents, tested either with or without rat liver S-9 microsomal preparation, resembled nonmutagenic drugs and not mutagenic carcinogens in the lymphocyte ^{3}H-thymidine assay (*15*). These data indicate, therefore, that the hypolipidemic agents fail to behave as electrophilic reactants capable of covalent interaction with cellular nucleic acid.

In preliminary work, we have tested plasticizers and related compounds for reactivity in both the Salmonella/microsome and the lymphocyte ^{3}H-thymidine assay. A dose-dependent increase in the number of *His*$^+$ revertant colonies was not observed over a wide concentration range of the plasticizers DEHP and di(2-ethylhexyl) adipate (DEHA) when tested in the absence or presence of rat liver S-9 microsomal mixture with *S. typhimurium* strains TA98 and TA100 (Table 1). The related compounds,

Table 1. *In vitro* assay of plasticizers with *Salmonella typhimurium* strains.[a]

		His⁺ revertants/plate[b]			
		TA98		TA100	
Compounds	Concentration, *M*	–S–9	+S–9	–S–9	+S–9
DEHP	10^{-2}	27	24	105	106
	10^{-4}	28	21	95	97
	10^{-6}	33	20	111	90
DEHA	10^{-2}	32	27	125	109
	10^{-4}	28	37	110	88
	10^{-6}	20	35	93	108
Ethyl-2-hexanol	10^{-2}	22	24	98	96
	10^{-3}	26	12	105	90
	10^{-4}	23	18	109	108
Ethylhexanoic acid	10^{-2}	22	18	91	90
	10^{-4}	35	21	101	94
	10^{-6}	21	27	103	122

[a] *S. typhimurium* TA98 and TA100 were incubated in the absence or presence of S-9 microsomal mixture on histidine-deficient agar with carcinogen or plasticizers for 2 days at 37°C.

[b] As a positive control, the indirect carcinogen 2-acetylaminofluorene (10 μg/plate) added in the presence of S-9 microsomal mixture was included in each experiment. In the experiment with strain TA98, 690 revertant colonies were counted per plate and with strain TA100, 353 revertant colonies per plate.

ethyl-2-hexanol and ethylhexanoic acid, were also without mutagenic activity in the Ames assay (Table 1). Both 2-ethylhexyl alcohol and 2-ethylhexanoic acid are potent peroxisome proliferators and it has been suggested that 2-ethylhexyl alcohol is the active part of the DEHP or DEHA molecule responsible for peroxisome proliferation (*4*). Also, 2-ethylhexyl alcohol is a metabolite of DEHP. In the lymphocyte ^{3}H-thymidine assay, DEHP, DEHA and ethyl-2-hexanol induced a dose-dependent inhibition of ^{3}H-thymidine incorporation into the replicating DNA of proliferating (concanavalin A-stimulated) C57BL/6J splenic lymphoid cells (Table 2). The dose-dependent inhibition of ^{3}H-thymidine incorporation into replicating DNA was accompanied by a dose-dependent increase in the ratio of [illegible] soluble to DNA-incorporated ^{3}H-thymidine in the plasticizer-treated lymphocytes (Table 2). This indicates that factors other than suppressed uptake of exogenous thymidine by plasticizer-treated lymphocytes are critical for the inhibition of ^{3}H-thymidine incorporation into DNA (*15*). To test whether the inhibition of ^{3}H-thymidine incorporation could be due to plasticizer-induced damage to lymphocyte DNA, plasticizer-treated lymphocytes were incubated in fresh culture medium without plasticizer. Previous work has established that inhibition of ^{3}H-thymidine into replicating lymphocyte DNA induced by DNA-damaging carcinogens such as methyl methanesulfonate (MMS) is not reversed by 3 hr incubation of carcinogen-treated lymphocytes in fresh medium without carcinogens (*18, 19*). However, inhibition of ^{3}H-thymidine incorporation by non-DNA-damaging drugs which act by metabolic mechanisms, such as hydroxyurea, can be reversed by 3 hr incubation of drug-treated lymphocytes in fresh medium without drug. As reported in Table 3, suppression of ^{3}H-thymidine incorporation into DNA by DEHP, DEHA and ethyl-2-hexanol was reversed by incubation of treated lymphocytes for 3 hr without the compounds. As with other peroxisome proliferators, therefore, DEHP and DEHA or related alcohol and acid analogs do not have the properties of electrophilic mutagens under standard *in vitro* conditions of testing.

The fundamental problem arises as to mechanisms by which peroxisome proliferators initiate cells for neoplastic transformation. A multienzyme pathway has recently been identified in rat liver peroxisomes for the β-oxidation of long-chain fatty acids (*20*), and peroxisome proliferators have been found to increase manyfold the capacity of rat liver for the β-oxidation of fatty acids (*21*). In particular, the administration of DEHP resulted in a substantial increase in peroxisomal palmitoyl-CoA oxidation in liver homogenates (*22*). A similar increase in the peroxisomal fatty acyl-CoA-oxidation system has been detected following the administration of other peroxisome proliferators, including clofibrate, nafenopin, Wy-14,643, tibric acid, gemfibrozil and methyl clofenapate (*5*). Peroxisomal fatty acyl-CoA oxidation appears to be independent of an electron transport chain and results in the production of H_2O_2 by direct transfer of electrons to O_2. A sevenfold increase in the rate of palmitoyl CoA-dependent H_2O_2 generation has been detected in the liver of rats administered nafenopin (*23*). Liver tumors as well as nontumor portions of liver in Wy-14,643-fed rats have also been found to demonstrate increased

Table 2. Amount of acid-soluble and DNA-incorporated ^{3}H-thymidine in splenic lymphoid cells treated with plasticizers.[a]

Compounds	Concentration, *M*	% of control ^{3}H-thymidine incorporation[b]		Ratio, acid-soluble ^{3}H-thymidine/ DNA-incorporated ^{3}H-thymidine[c]	
		–S-9[d]	+ S-9[e]	–S-9[d]	+ S-9[e]
None				0.78	0.41
DEHP	10^{-2}	55 ± 3		1.44	
	5×10^{-3}		41 ± 6		0.73
	10^{-4}	56 ± 10		1.33	
	5×10^{-5}		48 ± 7		0.66
	10^{-6}	64 ± 6		0.93	
	5×10^{-6}		71 ± 7		0.45
DEHA	10^{-2}	64 ± 9		1.40	
	5×10^{-3}		69 ± 2		0.52
	10^{-4}	89 ± 7		0.88	
	5×10^{-5}		93 ± 10		0.36
	10^{-6}	119 ± 12		0.63	
	5×10^{-6}		83 ± 4		0.42
Ethyl-2-hexanol	10^{-3}	28 ± 4		1.86	
	10^{-4}	108 ± 8		0.59	
	5×10^{-4}		59 ± 2		0.47
	5×10^{-5}		84 ± 5		0.39
	5×10^{-6}		92 ± 10		0.32

[a] Concanavalin A-stimulated C57BL/6J splenic lymphoid cells were incubated in the absence or presence of S-9 microsomal mixture in medium without or with plasticizer. Lymphoid cell DNA was labeled with 10 µCi ^{3}H-thymidine per culture during the final 30 min of incubation. Labeled cells were washed three times with phosphate-buffered saline, the labeled cells were transferred to clean glass tubes, and the cells were precipitated overnight at 4°C with 0.2*N* perchloric acid. Supernatant was then separated from precipitate by centrifugation at 850*g* for 20 min, radioactivity of acid-soluble ^{3}H-thymidine was measured in the 0.2*N* perchloric acid supernatant by liquid scintillation spectrometry, and DNA was extracted from the 0.2*N* perchloric acid precipitate for liquid scintillation measurement of DNA-incorporated ^{3}H-thymidine.

[b] Incorporation of ^{3}H-thymidine into DNA was measured for each lymphocyte culture as dpm/µg DNA. Values of ^{3}H-thymidine incorporation for control cultures of untreated lymphocytes were taken as 100%. Each reported value was obtained as the mean ± SD of three replicate cultures of treated lymphoid cells.

[c] Ratio of the mean values of acid-soluble to DNA-incorporated ^{3}H-thymidine obtained in three replicate lymphocyte cultures as dpm/µg DNA.

[d] Lymphoid cells were incubated for 1 hr without or with plasticizer.

[e] Cells were incubated for 2.5 hr without or with plasticizer plus 20% (v/v) S-9 microsomal mixture.

Table 3. Recovery of stimulated rates of ^{3}H-thymidine incorporation by splenic lymphoid cells treated with plasticizers.[a]

Compound	Concentration, *M*	% of control ^{3}H-thymidine incorporation[b]	
		0 hr	3 hr
Without S-9[c]			
MMS	10^{-3}	74 ± 8	32 ± 11
DEHP	10^{-5}	71 ± 10	96 ± 8
DEHP	10^{-3}	36 ± 4	58 ± 3
DEHA	10^{-2}	16 ± 8	55 ± 8
Ethyl-2-hexanol	10^{-4}	39 ± 1	105 ± 18
Hydroxyurea	10^{-4}	10 ± 1	110 ± 16
With S-9[d]			
DEHP	10^{-2}	22 ± 4	72 ± 17
DEHA	10^{-3}	24 ± 14	66 ± 9
Ethyl-2-hexanol	10^{-3}	13 ± 1	110 ± 22

[a] Concanavalin A-stimulated C57BL/6J splenic lymphoid cells were incubated in the absence or presence of S-9 microsomal mixture in medium with plasticizer, carcinogen, or hydroxyurea and were then incubated for the times indicated in fresh culture medium without plasticizer, carcinogen, or hydroxyurea. Lymphoid cell DNA was labeled with 10 µCi ^{3}H-thymidine per culture during the final 30 min of incubation.

[b] Incorporation of ^{3}H-thymidine into DNA was measured for each lymphocyte culture as dpm/µg DNA. Values of ^{3}H-thymidine incorporation for control cultures of untreated lymphocytes were taken as 100%. Each reported value was obtained as the mean ± SD of three replicate cultures of treated lymphoid cells.

[c] Lymphoid cells were incubated for 1 hr with carcinogen, plasticizer, or hydroxyurea prior to incubation in fresh culture medium without added carcinogen, plasticizer, or hydroxyurea.

[d] Cells were incubated for 2.5 hr with plasticizer plus 20% (v/v) S-9 microsomal mixture prior to incubation without plasticizer or S-9 microsomal mixture.

levels of both peroxisomal fatty acid β-oxidation and H_2O_2 (*24*). Increased H_2O_2 production by the proliferated peroxisomes could result in the production of OH· by the Haber-Weiss reaction catalyzed by cellular iron (*25*). It is suggested, therefore, that peroxisome proliferation in cells is accompanied by an increase in the rate of production and perhaps the steady-state levels of reactive oxygen species in the cells. Compatible with this proposal is the excessive accumulation of lipofuscin, indicative of increased lipid peroxidation, which occurs in liver parenchymal cells during Wy-14,643- (*24*) or methyl clofenapate- (*17*) induced liver tumorigenesis. Unstable oxygen species are highly reactive toward biological macromolecules, especially DNA. Oxidative degradation, strand breakage and crosslinking have been detected in isolated DNA upon interaction with H_2O_2 and OH· (*26*, *27*). Also, H_2O_2 has been found to induce DNA scissions in intact prokaryotic and eukaryotic cells (*28*, *29*) and chromatid breaks, chromatid exchanges and unscheduled DNA synthesis in intact mammalian cells (*30*, *31*). The important possibility arises, therefore, that sustained peroxisome proliferation initiates cells for neoplastic transformation by increasing intracellular production of DNA-damaging oxygen species.

Further changes in peroxisomal enzyme profiles induced by the peroxisome proliferators can be cited which could serve to increase cellular reactive oxygen species. First, the increase in peroxisomal catalase activity is disproportionately small compared to the increase in peroxisome volume (*32*) and H_2O_2-generating fatty acid β-oxidation (*33*) induced by the peroxisome proliferators. It will be recalled that catalase catalyzes the degradation of H_2O_2. Second, a substantial level of uricase activity has been detected in the peroxisomes of rodent liver (*34*). Although serum and tissue levels of uric acid have yet to be systematically measured in animals treated with peroxisome proliferators, increased levels of hepatic uricase would result in decreased levels of uric acid. Uric acid is a powerful antioxidant and a scavenger of oxygen radicals (*35*).

The occurrence of DNA damage by reactive oxygen species can be directly tested as a hypothesis for the initiation of cells in DEHP carcinogenesis. Such testing will include measurement of reactive oxygen formation, assessment of DNA damage (DNA strand breakage, DNA repair) by oxygen radicals, and examination of possible modulation by antioxidants (α-tocopherol, selenium) in phthalate ester hepatocarcinogenesis. In addition, work is required to examine possible covalent binding of isotopically labeled plasticizer to liver cell DNA, RNA and protein during chronic administration of plasticizer to rodents. Absence of consistent *in vitro* DNA-damaging activity does not necessarily exclude the formation of electrophilic metabolites from the plasticizers in liver of the intact animal. Finally, although no evidence has been reported to date for tumor promotion by the phthalate esters, the peroxisome proliferators Wy-14,643 and clofibrate have been observed to promote the appearance of hepatocellular carcinoma following initiation in F344 rats by diethylnitrosamine (*36*). Tumor promotion is a reversible process which, unlike initiation, appears not to depend upon covalent modification of biological macromolecules (*14*). However, mechanisms of tumor promotion are poorly understood, and proposed cellular/molecular mechanisms of promotion as of the date of this report (*14*) would add little to a basic understanding of phthalate ester carcinogenesis.

As a final note, we should stress that chemically induced peroxisome proliferation is not limited to rats and mice. Hepatic peroxisome proliferation has been induced by hypolipidemic drugs in hamsters, pigeons, chickens, cats, and rhesus monkeys (*37*, *38*). Also, ultrastructural changes in the liver of human subjects on long-term (6 months to 7 years) clofibrate therapy included an elevated number of microbodies (peroxisomes) (*39*). Consequently, the potential importance of peroxisome proliferator carcinogenesis impacts on many species including the human.

This work was supported by PHS Grant GM 23750 from the Department of Health and Human Services, Grant IN-123 from the American Cancer Society, and Grant 78-71 from the American Cancer Society, Illinois Division.

The authors gratefully acknowledge the excellent technical assistance of Matthew Connors and Ralph Conti.

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Environmental Health Perspectives
Vol. 45, pp. 41-50, 1982

Effects of Phthalate Esters on Lipid Metabolism in Various Tissues, Cells and Organelles in Mammals

by Frank P. Bell*

The effect of phthalate ester plasticizers on a variety of enzyme systems was studied in rats, rabbits and pigs. The feeding of di(2-ethylhexyl) phthalate (DEHP) to animals at levels from 0.1% to 1.0% in the diet resulted in diverse biochemical effects, such as inhibition of cholesterologenesis in liver, testes, and adrenal gland; inhibition of cholesterologenesis in brain and liver of fetal rats from DEHP-fed dams; decreased plasma cholesterol levels; decreased synthesis of hepatic phospholipid and triglyceride; increased fatty acid oxidation in isolated liver mitochondria; and a transient decrease in fatty acid oxidation in isolated heart mitochondria. The addition of DEHP to preparations of rat liver *in vitro* resulted in inhibition of cholesterologenesis, and its addition to isolated mitochondria from rat heart produced an inhibition of adenine nucleotide translocase. DEHP-feeding to rats and rabbits, however, did not affect platelet function as judged by collagen- and ADP-induced aggregation. The studies presented here indicate that the exposure of animals to phthalate esters can result in a significant perturbation of normal metabolism in liver, heart, testes, adrenal gland and brain and can affect blood lipid levels.

Introduction

A number of reviews (*1-5*) have thoroughly outlined the synthesis, properties, and industrial application of the phthalate esters in use today. The utility of the phthalates is so diverse that annual production in the USA alone is estimated at 1-2 × 10^9 lb with worldwide production reaching about 4 × 10[illegible] (*5*). The widespread use of phthalates and phthalate-containing products in all segments of society has, within the span of a few decades, resulted in global contamination by this class of compounds. The presence of phthalate esters in virtually all ecosystems (*5-17*) and in the tissues of man ([illegible]*22*), animals (*6, 7, 9, 11, 16, 23, 24*), and in the food supply of man (*24-26*) is a shocking revelation.

Phthalates were evaluated in the 1940s and 1950s by classical toxicological criteria and judged to have a low order of acute and chronic toxicity (*27-30*). It was not until the 1960s that the safety of the phthalates came into serious question. During the 1960s, scattered reports appeared in medical journals citing concern over the possible toxic effects of poly(vinyl chloride) (PVC) plastic medical devices which contained phthalate ester plasticizers (*31-36*). Concern over phthalate toxicity heightened with reports by Marcel and Noel (*37, 38*) and Jaeger and Rubin (*39, 40*) in 1970 that stored human blood was being contaminated with phthalate ester, namely, di(2-ethylhexyl) phthalate (DEHP), while stored in PVC plastic blood bags. It was subsequently shown that tissues from patients recently transfused and/or exposed to PVC plastic catheters accumulated detectable levels of DEHP in their tissues (*18-21*). During the same period (1970-1975), a wide variety of biological studies appeared in the literature which further challenged the safety of the phthalates (*24, 41-50*); these provided evidence that the phthalates could accumulate in mitochondria (*24*) and that phthalates were teratogenic (*41-43*), mutagenic (*44*), toxic to various types of cultured cells (*45-47*), capable of placental transfer (*48*), capable of altering hepatic ultrastructure (*49*) and capable of promoting hepatic lipid accumulation under certain conditions (*50*).

What was surprising to me, however, was that as

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late as 1975, the knowledge that had accrued concerning phthalate toxicity was devoid of studies on the effects of phthalate esters on biochemical pathways and events, which by necessity would underlie the qualitative toxic responses being observed. It was for this reason, in 1975, that efforts in my laboratory were directed towards investigating the possible effects of phthalate esters on lipid metabolism in various species. The observations presented in this paper summarize results of studies which were initiated in 1975 and which have continued up to the present time. The studies were conducted in rats, rabbits and pigs. Depending upon the nature of the study, phthalate esters were either fed as an admixture to the diets or added, *in vitro*, to tissue preparations. Details of the techniques and methods used throughout the studies have been described elsewhere and will be referenced in the table footnotes and figure legends. Statistical analyses were performed using Student's independent *t*-test.

Results and Discussion

Effects of Phthalate Esters on Sterol Metabolism

The reports that phthalate esters possess teratogenic and mutagenic potential (*41-44*) suggested the possibility of their interference with sterologenesis which is a key process in mammalian development. This, in fact, proved to be the case, and the evidence was first published in 1976 (*51, 52*).

Liver (Male Rats). An inhibition of cholesterol synthesis from ^{14}C-acetate is apparent within 48 hr after feeding 0.5% DEHP to male rats (Table 1). The inhibition is progressive and rises from 14% at 2 days to 70% by 11 days. Inhibition of labeled mevalonate incorporation into cholesterol by 0.5% DEHP feeding also occurs but is slower to develop; inhibition is not apparent until after 6 days of DEHP exposure. By 11 days, cholesterologenesis from ^{3}H-mevalonate is inhibited about 40%. The lag period in the development of inhibition of hepatic cholesterologenesis from mevalonate relative to acetate has been observed under other conditions which result in inhibition of cholesterologenesis (*54*). The incorporation of mevalonate into cholesterol can eventually be suppressed to the same extent as acetate incorporation, as revealed by livers from rats fed DEHP for 18 days (Table 1). The data in Table 1 suggest that the site of action of phthalate is at the level of HMG CoA reductase (3-hydroxy-3-methylglutaryl CoA reductase, EC 1.1.1.34) (*55*), the first rate-limiting step in hepatic sterol synthesis (*56*) (Fig. 1). Inhibition at this site would first affect the flow-through of acetate to cholesterol as demonstrated in Table 1 (and diagrammed in Fig. 1) and permit the inhibition to be circumvented initially by use of exogenous mevalonate as a substrate. The inhibition of sterologenesis from labeled mevalonate which develops later (after 6 days, Table 1) may be explained if one or more of the enzymes between mevalonate and cholesterol are substrate-induced and thereby decline in activity as mevalonate production diminishes.

Table 1. Effect of DEHP feeding on cholesterologenesis from ^{14}C-acetate and ^{3}H-mevalonate in liver minces from male rats.[a]

Level of DEHP in diet, %	Duration of feeding, days	Incorporation into cholesterol, % of control ^{14}C-Acetate	 ^{3}H-Mevalonate
0.5	0	100	[illegible]
	2	86	[illegible]
	4	63	[illegible]
	6	39	[illegible]
	11	30	[illegible]
1.0	18	29	[illegible]

[a] Male Sprague-Dawley rats (300-325 g) were fed Purina Chow (control) or Purina Chow containing di(2-ethylhexyl) phthalate (DEHP) (*52, 53*), at levels of 0.5% or 1.0% (w/w). Liver minces were prepared and incubated for 3 hr in 4.0 ml (0.5% study) or 3.7 ml (1.0% study) of Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 4 μCi sodium acetate-1-^{14}C (SA 58 mCi/mmole) and 2.4 μCi DL-mevalonic-5-^{3}H acid (DBED salt, SA 6.7 Ci/mmole) (*52*). After incubation, the samples were hydrolyzed (saponified) with alcoholic KOH, and then the neutral sterols were extracted with *n*-hexane and subsequently fractionated by thin-layer chromatography (*52*). Values are means of two or three DEHP-fed animals expressed relative to control values obtained in parallel experiments.

In all studies, the decreased synthesis of cholesterol was paralleled by decreases in synthesis of lanosterol and squalene (*52*), as would be anticipated from Figure 1. Additionally, the cholesterol content of livers from rats fed 0.5% DEHP for 6 days was not different from that of control rats (*52*).

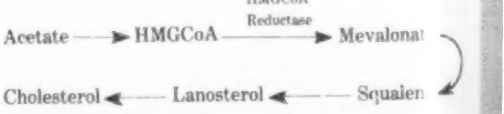

FIGURE 1. Cholesterol, the principal C_{27} sterol of liver, is derived from units of acetyl CoA via a sequence of 2 or more intermediates (*57*) which include 3-hydroxy-3-methylglutaryl CoA (HMG CoA), mevalonic acid, squalene (a C_{30} hydrocarbon) and lanosterol (a C_{30} sterol). The conversion of HMG CoA to mevalonate by HMG CoA reductase (EC 1.1.1.34) is the primary rate-limiting step in the formation of squalene and sterols (*56*). Secondary control sites beyond mevalonate may also function (*54, 56-58*).

Table [illegible]. ^{14}C-Acetate incorporation into sterols of normal male [illegible] liver minces: inhibition by exogenously added DEHP.[a]

DEHP concn., [illegible]	n	^{14}C-Acetate incorporation into digitonin-precipitable sterols, % of control
[illegible]	9	100 ± 0
[illegible]	6	92 ± 15
[illegible]	6	75 ± 12
[illegible]	4	87 ± 7
[illegible]	8	54 ± 6

[a] Livers from normal male Sprague-Dawley rats (275-300 g, maintained on Purina Chow) were used to prepare minces (500 mg) (5[illegible]) which were incubated for 3 hr in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4. Assays were initiated by the addition of 2 µCi sodium acetate-1-^{14}C (SA 58.3 Ci/mole) and DEHP. DEHP was added as a Tween-20-stabilized dispersion (*60*). After incubation, the samples were hydrolyzed with alcoholic KOH (*52*). The nonsaponifiable lipids were recovered by extraction with *n*-hexane (*52*) and the 3β-OH sterols (principally cholesterol) were precipitated with digitonin and assayed for radioactivity (*52, 59*). Values are means ± SEM of the number of animals designated by *n*.

thus indicating that DEHP-induced inhibition of hepatic cholesterologenesis is unlikely to be attributed to end product inhibition. In fact, feeding of phthalates for longer periods (21 days) tends to reduce hepatic cholesterol (*55*). End product inhibition is also inconsistent with the observation that DEHP inhibits ^{14}C-acetate incorporation into sterols when added directly to normal male rat liver minces (Table 2).

Liver (Female Rats). Studies in female rats fed 1.0% DEHP for 13 days (*61*) indicated that inhibition of hepatic sterologenesis is not sex-specific (Table [illegible]).

Liver (Male Rabbits). Inhibition of sterologenesis in liver from rabbits fed DEHP (*59*) also dismissed the possibility that the effect of DEHP is species-specific for the rat (Table 4).

Table [illegible]. Effect of DEHP feeding on the incorporation of ^{14}C-m[illegible]nate into sterols and squalene by liver minces from female rats.[a]

	Incorporation, dpm/g wet wt	
	C_{27} + C_{30} sterols	Squalene
Control	40520 ± 2440	56870 ± 5175
1% DEHP	29635 ± 3645	24355 ± 2490
	$p < 0.05$	$p < 0.001$

[a] Female Sprague-Dawley rats (245-255 g) were fed either Purina Chow or Purina Chow supplemented with 1.0% DEHP (w/w) for 13 days. Liver minces (500 mg) were incubated for 3 hr at 37°C in 3.4 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 1 µCi DL-mevalonic-2-^{14}C acid (DBED salt, SA 40.8 Ci/mole). After incubation, the samples were hydrolyzed with alcoholic KOH, the nonsaponifiable lipids were extracted with *n*-hexane (*52*) and fractionated by thin-layer chromatography to yield C_{27} sterols (principally cholesterol), C_{30} sterols (principally lanosterol) and squalene (*52*). Values are means ± SEM of six animals per group.

Table 4. Effect of DEHP feeding on the incorporation of ^{3}H-mevalonate into sterols and squalene by rabbit liver minces.[a]

	Incorporation, dpm/g wet wt		
	C_{27} sterol	C_{30} sterols	Squalene
Control	28890 ± 6880	9115 ± 2610	33080 ± 11360
1% DEHP	12140 ± 2815	3945 ± 655	13675 ± 5490
	$p < 0.05$	$p < 0.01$	

[a] Male New Zealand rabbits (2.5-3 kg) were fed Purina Chow Pellets or 1% DEHP (w/w) as an admixture in Purina Chow Pellets for 28 days. Liver minces (500 mg) were prepared and incubated for 3 hr at 37°C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 0.25 µCi DL-mevalonic-5-^{3}H(N) acid (DBED salt, 5 Ci/mmole). After incubation, the samples were treated with alcoholic KOH to hydrolyze the tissue and to saponify the lipids. The neutral sterols and squalene were extracted with *n*-hexane and subsequently fractionated by thin-layer chromatography. Values are means ± SEM of six animals.

Adrenal and Testes. Inhibition of sterologenesis in DEHP-fed animals is not restricted to the liver. The results of Table 5 show that adrenal glands and testes are similarly affected (*59, 61*), with inhibition reaching 20-25% of normal synthesis. Impaired sterologenesis in adrenal and testicular tissue may, in part, be responsible for the developmental abnormalities (*62*) reported to occur in fetuses from phthalate-treated dams (*41, 42, 46*) and for testicular atrophy in young and adult animals (*63-67*).

Developing Fetuses and Suckling Offspring. Placental transfer of DEHP has been reported in rats (*48*). The exposure of the developing fetus to DEHP by this route may have important implications in the development of the fetus. The results of Table 6 indicate that fetuses taken by cesarean section from pregnant rats fed DEHP beginning 5 to 10 days after conception have an impairment of sterologenesis in brain and liver (*68*). Furthermore, if dams exposed to DEHP during gestation, and during the postnatal period as well, are permitted to nurse their naturally born offspring, hepatic sterologenesis in the pups is reduced below control values (Table 7). In 8-day suckling rats, hepatic sterologenesis was reduced 20% below control values and was accompanied by increased liver size and decreased body weight (Table 7). The decreased body weight seen in the 8-day-old pups was not apparent in litters permitted to reach weaning age (21 days) (Table 8). DEHP feeding to the dams resulted in a significant lowering of plasma cholesterol ($p < 0.02$) but did not affect plasma cholesterol levels in the pups (Table 8).

Table 5. Effect of DEHP feeding on the incorporation of ^{14}C-mevalonate into C_{27} + C_{30} sterols and squalene in rat adrenal gland and testis.[a]

Organ	Diet	Duration of study, days	Incorporation, dpm/100 mg wet wt	
			Sterols	Squalene
Adrenal (♀)	Control	13	352 ± 23	124 ± 10
			$p < 0.01$	NS
	1% DEHP	13	266 ± 8	105 ± 7
Adrenal (♂)	Control	73	322 ± 20	84 ± 4
			$p < 0.02$	NS
	1% DEHP	73	264 ± 12	66 ± 10
Testis	Control	28	233 ± 15	212 ± 9
			$p < 0.02$	$p < 0.01$
	0.5% DEHP	28	175 ± 12	149 ± 13

[a] Sprague-Dawley rats of both sexes were fed Purina Laboratory Chow or the chow diet supplemented with DEHP. Adrenal glands were removed from females (body wt 251 ± 3 g, $n = 12$) and males (body wt 320 ± 20 g, $n = 10$), dissected free of adhering tissue and capsular material, and then incubated for 3 hr at 37°C in 1.52 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 1.0 μCi DL-mevalonic-2-^{14}C acid (DBED salt, 40.8 Ci/mole). Testes (right side) from rats weighing 225-250 g were decapsulated and incubated as the whole gland for 3 hr at 37°C in 5.0 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 2.5 μCi DL-mevalonic-2-^{14}C acid (DBED salt, 40.1 Ci/mole). After incubation, the tissues were treated with alcoholic KOH as described in the footnote to Table 1. The neutral sterols and squalene were extracted with n-hexane and subsequently fractionated by thin-layer chromatography. Values are means ± SEM (male adrenals, $n = 5$ per group; all others, $n = 6$ per group).

Table 6. DEHP feeding to rats during gestation: effect on the incorporation of radiolabeled acetate and mevalonate into cholesterol in fetal brain and liver.[a]

Expt. no.	Treatment	Labeled compound	Incorporation of labeled compound into cholesterol		
			^{14}C-Acetate dpm/brain	^{14}C-Mevalonate, dpm/brain	^{3}H-Mevalonate, dpm/mg dry wt of liver
1	Pregnant rats were fed 0.5% DEHP for 13 days beginning on the 5th day of gestation. Fetuses were taken by cesarean section on the 18th day of gestation. Brains were obtained from seven fetuses from seven different dams. Livers were obtained from fetuses from up to eight dams; not more than two fetuses per dam were used.	Control	— —	13570 ± 700 $n = (7)$	6680 ± 300 $n = (14)$
		DEHP	—	11510 ± 520 $n = (7)$ $p < 0.05$	5675 ± 220 $n = (13)$ $p < 0.02$
2	Pregnant rats were fed 1.0% DEHP for 8 days beginning on the 10th day of gestation. Fetuses were taken by cesarean section on the 18th day of gestation. Brains and livers were obtained from fetuses from two to three different dams.	Control	25300 ± 1795 $(n = 8)$	—	4990 ± 490 $(n = 9)$
		DEHP	18375 ± 2240 $(n = 6)$ $p < 0.05$	—	3010 ± 170 $(n = 6)$ $p < 0.01$

[a] Livers and brains were incubated as the entire tissue for 3 hr at 37°C in 3.5 ml Krebs-Ringer-bicarbonate buffer, pH 7.4. Radioactive precursors (acetate-l-^{14}C, sodium salt, SA 59 Ci/mole, DL-mevalonic acid-2-^{14}C, DBED salt, SA 46 Ci/mole, and DL-mevalonic acid-5-^{3}H, DBED salt, SA 5 Ci/mmole) were present at a level of 2μCi.

Plasma Cholesterol. Certain phthalate esters evoke a hypocholesterolemic response in rats of either sex (Tables 8 and 9). At levels of 0.5% and 1.0% in the diet, DEHP reduces plasma cholesterol about 20% in the male rat (Table 9) (*55, 59, 69*); this reduction appears to represent a maximum response inasmuch as 0.5% and 1.0% DEHP appear to have equivalent effects (*55, 59, 69*). The maximum cholesterol-lowering effect occurs within the first week of DEHP feeding (*55*). Since a hypocholesterolemic effect of phthalates has also been observed in mice (*70*) but not in rabbits (*59, 69*), this response appears to have some degree of species specificity.

Comparison of Dimethyl Phthalate (DMP), Di-n-butyl Phthalate (DBP) and Di-2-ethylhexyl Phthalate (DEHP). In rats fed DMP, DBP or DEHP at a level of 25 mmole/kg diet (equivalent to 1.0% DEHP) for 21 days, DEHP and DBP reduced plasma cholesterol levels 16% and 2, respectively, and inhibited hepatic cholesterologenesis from labeled mevalonate by 67% and 30%, respectively (Table 9). It is possible that the hypocholesterolemic effect of DBP and DEHP is a direct consequence of the inhibition of hepatic sterologenesis, although other mechanisms could be possible. Since inhibition of hepatic sterologenesis from labeled mevalonate (Table

Table Incorporation of ^{14}C-mevalonate into sterols in livers of 8-day-old suckling rats from dams fed 0.5% DEHP during gestation and throughout the postnatal period.[a]

	Pup wt, g	Liver wt, % body wt	Incorporation into C_{27} + C_{30} sterols, dpm/g wet wt × 10^{-2}
Control	21.7 ± 0.3	2.75 ± 0.03	3906 ± 280
DEHP	18.8 ± 0.7	3.01 ± 0.06	3107 ± 208
	$p < 0.01$	$p < 0.01$	$p < 0.05$

[a] Pregnant rats were fed 0.5% DEHP in the diet (w/w) beginning on the 5th day of gestation and were permitted to deliver and to nurse the pups for 8 days; the DEHP diet was continued throughout the entire postnatal period. Liver minces were prepared from each pup and incubated 3 hr at 37°C in 3.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 2μCi DL-mevalonic-5-^{3}H acid, (DBED salt, SA 5 Ci/mmole). Control pups (n = 10) were from 1 dam; pups in the DEHP group (n = 12) came in equal numbers from two dams.

Table 8. Plasma cholesterol levels in DEHP-fed nursing dam rats and their suckling pups.[a]

	n	Body wt, g	Total plasma cholesterol, mg/dl
Controls			
Dams	4	363 ± 6	61 ± 5[b]
Pups	41	46 ± 1	102 ± 2
DEHP-fed			
Dams	4	353 ± 6	37 ± 6[b]
Pups	26	48 ± 1	102 ± 4

[a] Female rats were fed Purina Laboratory Chow or the chow supplemented with 0.5% DEHP (w/w) from the first day of gestation until the pups were weaned at 21 days. Cholesterol was measured as described (*59*). Values are means ± SEM of the number of animals designated by n.

[b] Significant, $p < 0.02$.

Table 9. Effect of feeding various phthalate esters on lipid metabolism in the rat.[a]

	Liver weight, % body wt	Total liver lipid, mg/g wet wt	Liver cholesterol, mg/g wet wt	Total plasma cholesterol, mg/dl	Incorporation of ^{14}C-mevalonate into cholesterol in liver, % of control
Control	4.0 ± 0.1	42.7 ± 1.4	4.5 ± 0.2	87 ± 5	100
DMP	4.1 ± 0.2	38.5 ± 1.2[b]	3.1 ± 0.2[c]	80 ± 3	114 ± 21
DBP	4.6 ± 0.1[c]	47.3 ± 2.2	3.8 ± 0.2	69 ± 3[b]	70 ± 6[b]
DEHP	6.8 ± 0.2[c]	44.2 ± 1.0	4.1 ± 0.1	73 ± 5[b]	33 ± 8[c]

[a] Male Sprague-Dawley rats were fed Purina Chow (control) or the chow containing dimethyl phthalate (DMP), di-n-butyl phthalate (DBP), or di(2-ethylhexyl) phthalate (DEHP); the phthalates were present at a level of 2.5 mmole/100g feed. The diets were fed for 21 days. Details of the analyses have been presented (*55*). All values are means ± SEM of 5-10 observations.

[b] $p < 0.05$, Student's independent t-test.

[c] $p < 0.01$, Student's independent t-test.

9) and labeled acetate (*55*) is greater with DEHP than DBP and since DMP does not have this action (Table 9) (*55*), the presence of a two-carbon alkyl chain appears to be the minimum requirement for inhibition of sterologenesis.

DMP differed from DBP and DEHP in two other respects; it reduced liver total lipid and liver total cholesterol (Table 9), whereas DBP and DEHP showed no significant effect on these parameters. Additionally, DMP did not produce hepatomegaly as did DBP and DEHP (Table 9). The increased liver size in the DBP- and DEHP-fed animals appeared to represent a true increase in tissue mass rather than an edematous change since the ratio of lipid-free dry weight to wet weight of liver was similar to control values (*55*).

Effects of DEHP Feeding on Biosynthesis of Fatty Acids and Glyceryl Lipids in the Rat

Feeding DEHP to rats affects not only sterol synthesis as mentioned previously, but synthesis of other lipids as well. Interestingly, not all tissues appear to be affected to the same extent or in the same manner. The incorporation of 1-^{14}C-acetate into fatty acids, phospholipids and triglycerides was not statistically significantly altered, *in vitro*, in heart and aorta of male rats fed 0.5% DEHP for 10 days (Table 10). By contrast, 1-^{14}C-acetate incorporation into these same lipids was reduced about 40% in liver; total ^{14}C-labeled lipid synthesis (includes sterols) was also reduced about 48% (Table

Table 10. Effect of DEHP feeding (0.5%) on the incorporation of ^{14}C-acetate into fatty acids and glyceryl lipids in various tissues of the rat, *in vitro*.[a]

Tissue	Group	^{14}C-Acetate incorporation, dpm/g wet weight			
		Phospholipid	Triglyceride	Free fatty acid	Total lipid
Aorta	Control	320 ± 105	295 ± 150	290 ± 120	1225 ± 4[illegible]
	DEHP	205 ± 75	315 ± 175	200 ± 60	965 ± 3[illegible]
Heart	Control	1765 ± 165	1095 ± 90	1895 ± 200	6755 ± 4[illegible]
	DEHP	1500 ± 145	1085 ± 135	1515 ± 80	6245 ± 4[illegible]
Kidney	Control	2470 ± 220	1945 ± 220^b	1775 ± 145	8400 ± 4[illegible]
	DEHP	2060 ± 130	1315 ± 100^b	2185 ± 120	8095 ± 3[illegible]
Liver	Control	695 ± 80^c	625 ± 105^b	945 ± 140^c	3525 ± 4[illegible]^c
	DEHP	425 ± 40^c	370 ± 65^b	500 ± 60^c	1860 ± 1[illegible]^c
		$p < 0.02$	$p < 0.05$	$p < 0.02$	$p < 0.0$[illegible]

[a] Male Sprague-Dawley rats were fed Purina Chow (control) or the chow containing 0.5% DEHP (w/w) for 10 days. Tissue slices were prepared from liver, heart and kidney and incubated for 3 hr at 37°C in 5.0 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, containing 4 μCi sodium acetate-1-^{14}C (SA 58.6 mCi/mmole) (*53*). Aortas were incubated under exactly the same conditions with the exception that the tissue was bisected longitudinally rather than sliced. All values are means ± SEM of six animals.

[b] Significant, $p < 0.05$.

[c] Significant, $p < 0.02$.

10). In kidney, ^{14}C-acetate incorporation in triglycerides was reduced 33%, whereas synthesis of the fatty acids and phospholipids was not significantly altered by DEHP feeding (Table 10). Fractionation of the hepatic phospholipids synthesized from ^{14}C-acetate in rats fed 0.5% DEHP showed a significant reduction in the distribution of ^{14}C-acetate into the phosphatidylcholine fraction of the liver phospholipids (*53*). In addition, feeding 0.5% DEHP to male rats for 9 days resulted in 37-44% reduction in the esterification of ^{3}H-oleate into diglycerides and triglycerides in liver minces (*55*). The above studies suggest that DEHP can, in certain tissues, inhibit de novo fatty acid synthesis, decrease fatty acid esterification, and selectively affect the synthesis of specific phospholipid species; the latter has been confirmed chemically as well (*17*).

Effects of DEHP on Mitochondrial Function

The ability of DEHP to accumulate in heart tissue of animals (*23*, *24*) and man (*21*) and, more specifically, to accumulate in the mitochondrial fraction of heart tissue (*23*, *24*) suggested a need to investigate possible biochemical effects of DEHP on mitochondrial function.

Fatty Acid Oxidation in Heart and Liver Mitochondria from Rat, Rabbit and Pig. DEHP feeding to rats, rabbits and pigs resulted in an increased capacity of their liver mitochondria to oxidize palmitic acid (*72*). In suitably prepared suspensions of isolated mitochondria, the oxidation of ^{14}C-palmitoyl CoA was enhanced two- to threefold after several days' exposure to the DEHP (Table 11). The enhanced capacity to oxidize ^{14}C-palmitoyl CoA substrate was undiminished throughout the longest periods of exposure studied (i.e., 12 days in rabbits and 35 days in rats). In the rat, the maximum response appeared at a level of 0.1% DEHP in the diet (Table 11). The response of heart mitochondria to DEHP feeding was markedly different from that shown in liver. Ingestion of 0.1% DEHP for 2 days resulted in a 25% reduction in fatty acid oxidation by heart mitochondria. The inhibition increased to about 50% in 3 to 4 days (Table 11). The inhibition, however, appears to be a transient effect, since full oxidizing capacity is restored in 8 or 9 days even though DEHP-feeding is continued. The addition of DL-carnitine and cofactors to heart mitochondria from rats fed DEHP for 5 days resulted in a stimulation of ^{14}C-palmitoyl CoA oxidation (Table 12). This suggests that the inhibition is not a result of functional change in the fatty acid oxidation system of the mitochondria.

Mitochondrial Adenine Nucleotide Translocase. Adenine nucleotide translocase is one of the mitochondrial anion translocators. It is located in the inner mitochondrial membrane and is responsible for affecting a mole:mole exchange of extramitochondrial ADP for intramitochondrial ATP. It is via this specific mechanism that ATP generated by oxidative phosphorylation can be transported into the cytoplasma to support cytosolic energy-requiring reactions. The addition of DEHP to suspensions of isolated heart mitochondria results in an inhibition of adenine nucleotide translocase. Activity of the transporter was reduced 37% by 300 μ*M* DEHP (Fig. 2). Such an effect of DEHP may be the mechanism underlying the death of myocardial cells cultured in the presence of DEHP (*45*, *74*) and

Table Effect of oral ingestion of DEHP on the oxidation of ^{14}C-palmitoyl CoA in isolated heart and liver mitochondria.[a]

Tissue	Species	Dose	Duration, days	Dpm $^{14}CO_2$/mg protein, % of control	No. of observations
Heart	Rat	0.1% in diet	2	77	1
			3	55 ± 25	3
			4	55	2
			9	110	1
	Rat	0.5% in diet	4	61	2
			9	103 ± 14	6
	Rat	1.0% in diet	9	98	2
			16	105 ± 5	5
Liver	Pig	0.8 mg/kg per os, b.i.d.	2	170	2
	Rabbit	1.0% in diet	12	236 ± 77	4
	Rat	0.1% in diet	2	284	1
			3	191 ± 1	3
			4	299	1
	Rat	0.5% in diet	4	548	2
			8	272 ± 16	3
			10	199 ± 21	3
			16	329	2
	Rat	1.0% in diet	16	227 ± 15	3
			35	278	2

[a] Mitochondria were isolated as previously described (*72*) and incubated for 3 hr at 37°C in the presence of 1-^{14}C-palmitoyl CoA (SA 56 Ci/mole). Oxidation of ^{14}C-palmitoyl CoA was assessed by measuring $^{14}CO_2$ production (*72*).

Table 12. Stimulation of ^{14}C-palmitoyl CoA oxidation by carnitine and cofactors in heart mitochondria from DEHP-fed rats.[a]

Additions to incubation medium	$^{14}CO_2$ production, dpm/mg mitochondrial protein
None	2100
DL-Carnitine	2740
ATP, CoA-SH	4660
ATP, CoA-SH, DL-carnitine	5710

[a] Male Sprague-Dawley rats (225-250 g) were fed Purina Laboratory Chow containing 1.0% DEHP (w/w) for 6 days. Mitochondria were isolated from pools of two hearts, and all assays were performed in triplicate (see footnote to Table 11) in a total volume of 7.0 ml (*72*). When present, the levels of the various cofactors in the incubation mixtures were: ATP, 15 mg; CoA-SH, 4 mg; NaF, 0.8; MgCl·6H_2O, 3 mg. DL-Carnitine ·HCl was added to a final concentration of 2.6 *mM*.

also be related to the decrease in spontaneous heart rate that is observed in rat hearts perfused with DEHP-containing medium (*75, 76*). Despite the fact that DEHP accumulates in the hearts of DEHP-fed rats ([illegible]), we did not observe a change in adenine nucleotide translocase activity in mitochondria isolated from hearts of rats fed 0.5% DEHP for up to 10 days (*73*). The fact that DEHP inhibited adenine nucleotide translocase when added directly to mitochondria, but not when fed to the rats, may indicate that the level of DEHP accumulating in the hearts during the 10-day feeding trials was insufficient to significantly affect the enzyme.

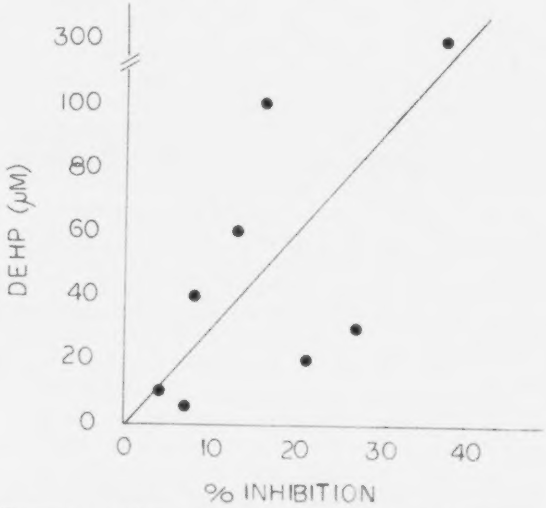

FIGURE 2. Inhibition of mitochondrial adenine nucleotide translocase. Mitochondria were isolated from normal male rat heart and assayed (*73*) in the presence of various concentrations of DEHP. Values are means of 15 animals at 20, 40 and 60 *M* DEHP and means of three to four animals at all other levels.

Effect of DEHP Feeding on Platelet Lipid Synthesis and Function in Rats and Rabbits

The well-known fact that blood and blood products become contaminated with DEHP during stor-

pages tightly bound

Table 13. Effect of DEHP feeding on platelet function and prothrombin time.[a]

Species	Group	Platelet number × 10^6/mm^3	Aggregation		Prothrombin time, sec
			Collagen-induced	ADP-induced	
Rat	Control	1.57 ± 0.03	30.7 ± 6.1	27.8 ± 1.9	12.7 ± 0.3
	DEHP-fed	1.66 ± 0.09	35.7 ± 4.9	30.2 ± 2.6	11.6 ± 0.3
Rabbit	Control	0.61 ± 0.05	18.0 ± 3.6	–	5.5 ± 0.[illegible]
	DEHP-fed	0.60 ± 0.04	17.9 ± 1.9	–	5.6 ± 0.1

[a] Male New Zealand rabbits (2.5-3 kg) and male Sprague-Dawley rats received Purina Chow or the chow supplemented with DEHP. Rats (150-160 g at beginning of study) received 0.5% DEHP for 32 days and rabbits received 1.0% DEHP for 28 days. All methods used are detailed elsewhere (*69*). Values are means ± SEM of six animals in each control group, and seven animals in each treated (DEHP) group.

age in PVC plastic storage bags has caused great concern because of the uncertainty as to what DEHP may do to blood cell physiology (*77*). For example, it has been reported that DEHP may cause increased platelet aggregation in blood stored in PVC storage bags (*45*), increased platelet adhesion to artificial surfaces (*78*) and decreased survival of platelets stored in the frozen state in PVC bags (*79*). In view of the above, we studied the function of platelets exposed to DEHP *in vivo* through a regimen of DEHP feeding. DEHP was fed in the diets of rats (0.5%) and rabbits (1.0%) for 32 and 28 days, respectively. At the end of the feeding period, platelet function was studied. Synthesis of phospholipids and sterols by the platelets was also studied because of the importance of these two lipid classes to membrane structure and stability (*80*), particularly since platelet aggregation is a membrane-dependent phenomenon. DEHP feeding did not affect phospholipid synthesis from acetate-1-^{14}C, or sterol synthesis from mevalonic-5-^{3}H acid, in platelets from young (6-week-old) or old (5-month-old) rats (*69*). Additionally, the feeding of DEHP did not significantly ($p > 0.05$) alter platelet number, collagen-induced platelet aggregation or ADP-induced aggregation in either the rabbit or rat (Table 13). Prothrombin time was also essentially unchanged (Table 13). The results suggest that the increased sensitivity of platelets exposed to DEHP *in vitro* (*45, 78, 79*) does not accurately reflect their response to DEHP exposure *in vivo*.

Conclusions and Suggestions

The data presented here make it abundantly clear that the exposure of animals to phthalate esters can result in a significant perturbation of normal metabolic patterns in liver, heart, testes, adrenal gland and brain and can affect blood lipids (*51-53, 55, 59-61, 68, 69, 72, 73, 77*). The ability of phthalates to alter lipid metabolism in the developing fetus and in suckling animals is also apparent and becomes a point of particular concern. At the time of this writing, no published biochemical studies similar to those reported here have been conducted in tissues from subhuman primates or man; such studies are urgently needed in order to assess more accurately the potential hazard of the phthalates to human health. At the present time, the concern of the medical profession over the exposure of patients to phthalates while undergoing medical treatment has attracted widespread attention in government and industry. Hopefully, the concern over the use of phthalate-containing devices in the field of medicine will attract greater attention to the more important issue of what potential health hazards mankind is exposed to as a result of involuntary chronic exposure to phthalates in the environment. Evidence that chronic exposure of man to DEHP may result in neurological disorders has been reported (*81*). A further point to be made is a suggestion that pharmacokinetic studies with phthalates should be less preoccupied with tissue distribution and more concerned with subcellular distribution; the reasoning follows. Much of the work described here has been concerned with biochemical systems dependent upon the activity of membrane-bound (particulate) enzymes—e.g., sterol synthesis (HMG CoA reductase), adenine nucleotide translocase, and fatty acid oxidation. Membrane-bound enzymes, in general, are sensitive to changes in membrane fluidity which reflects alterations in the physical state of the membrane-lipids (*82, 83*). The possibility exists that the effects of phthalates (inhibitory or stimulatory) on various membrane-bound enzymes is an indirect effect of their ability to modify membrane fluidity. Being lipophilic, the phthalates can partition across organellar membranes and perturb the composition and physical state of the lipid microenvironment (domain) surrounding certain particulate enzymes. Since the response of specific membrane-bound enzymes to changes in membrane fluidity cannot be determined *a priori*, it is not inconsistent that phthalate exposure should inhibit some enzymes while stimulating others. Consequently, pharmacokinetic studies would yield information of greater potential value if distribution data were obtained for specific cell fractions (endoplasmic reticulum, mitochondria, cytosol, etc.) rather than for total tissue. The growing knowledge of factors regulating particulate enzymes makes it clear that relatively low levels of a chemical agent in tissue may be misleading in terms of toxicity potential, since levels considered to be inconsequential on a tissue or organ basis may be disastrously high on an organellar basis.

The author gratefully acknowledges the assistance of the following people during the conduct of these studies: B. Brundage, P. Gillies, E. Hubert, M. Makowske, R. Mendoza, D. Nazir, E. Nishizawa, W. Nusca, W. Phillips, C. Patt, D. Schneider and S. Wang.

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Environmental Health Perspectives
Vol. 45, pp. 51-56, 1982

Mitochondrial Toxicity of Phthalate Esters

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The effects of mono- and dibutyl phthalate and mono- and di(2-ethylhexyl) phthalate on energy-dependent K^+ uptake, respiration rates, and succinate cytochrome c reductase activities of isolated rat liver mitochondria were evaluated. The energy-coupling processes, active K^+ transport and oxidative phosphorylation, were affected most by di-*n*-butyl phthalate and mono(2-ethylhexyl) phthalate. Mono-*n*-butyl phthalate had a moderate effect on energy coupling and di(2-ethylhexyl) phthalate had no apparent effect. The potency of inhibition of succinate cytochrome c reductase activity was mono(2-ethylhexyl) phthalate > di-*n*-butyl phthalate > mono-*n*-butyl phthalate = di(2-ethylhexyl) phthalate. It is concluded that phthalate esters affect mitochondrial activities by altering the permeability properties of the inner membrane and by inhibiting succinate dehydrogenase activity.

Introduction

The widespread distribution of phthalate ester plasticizers in the environment and the detection of significant quantities of di(2-ethylhexyl) phthalate (DEHP) in human blood stored in poly(vinyl chloride) bags (*1*) have led to concern about the possible biological effects of these compounds. An important observation is found in the NCI/NTP bioassay report (*2*) that DEHP is a hepatocarcinogen in B6C3F$_1$ mice and Fischer 344 rats. Hepatic changes have been reported after both oral and intraperitoneal administration of DEHP. One specific *in vivo* effect that has been identified is the decrease in mitochondrial succinate dehydrogenase activity (*3, 4*). In addition, swollen liver mitochondria were observed in rats (*7*) after oral administration of DEHP or mono(2-ethylhexyl) phthalate (MEHP). Studies *in vitro* have revealed that the oxidative and phosphorylation processes of rat liver mitochondria are impaired by phthalate esters (*5-7*). Takahashi (*6*) studied the effects of dialkyl and monoalkyl phthalate esters on mitochondrial respiration rates with succinate as the oxidizable substrate. Respiratory control ratios (which are indicative of coupling between oxidative and phosphorylation activities) of isolated rat liver mitochondria decreased most with dialkyl phthalates that contain alkyl chains of three or four carbons, i.e., di-*n*-propyl and di-*n*-butyl phthalates. DEHP had no apparent effect on respiratory control. For the monoalkyl phthalates, respiratory control ratios decreased as the alkyl chain was increased up to seven carbons. MEHP was one of the most effective phthalate esters in reducing the respiratory control ratio.

We sought to explain the mechanism(s) by which phthalate esters interfere with mitochondrial activities. A comparative study was conducted on the effects of mono-*n*-butyl phthalate (MBP), di-*n*-butyl phthalate (DBP), MEHP and DEHP on energy coupling and electron transport activities of isolated rat liver mitochondria.

Materials and Methods

Mitochondria were isolated fresh daily from the livers of 250-g male rats of the CD strain, as described by Stancliff et al. (*8*). Protein concentrations were determined by the Lowry method (*9*) with the use of bovine serum albumin as a standard.

Respiratory rates were measured with a Clark-type oxygen electrode (*10*) in 2 ml of a medium consisting of 250 mM sucrose, 10 mM KCl, 10 mM Tris HCl (pH 7.6), 3 mM NaH_2PO_4, 5 mM $MgCl_2$, and 1.8 µg/ml rotenone. Mitochondrial samples (3 mg protein) were incubated for 2 min with the phthalate esters prior to the addition of substrate. Respiration was initiated by the addition of succinate (2.0 mM final concentration), and phosphorylation was initiated by the addition of 0.25 µmole of ADP. The control samples had the same final concentration of ethanol (0.24%) that resulted from the addition of the phthalate esters.

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Volume changes of mitochondria due to active K$^+$ uptake were measured (*11*) with a Beckman Acta III recording spectrophotometer at 540 nm. The concentrations of the components of the reaction medium were 100 *mM* sucrose, 28.6 *mM* potassium acetate, 5 *mM* Tris-acetate (pH 7.6), 1.2 μg/ml rotenone, 0.02 μg/ml valinomycin, and either 2.0 *mM* succinate, 2.0 *mM* ascorbate + 0.15 *mM* *N,N,N',N'*-tetramethyl-*p*-phenylenediamine(TMPD), or 2.0 *mM* ATP. Mitochondrial samples (2 mg protein) were added to 3 ml of reaction medium and incubated with the phthalate esters for 2 min prior to the addition of the energy source.

Succinate cytochrome c reductase activity was measured (*12*) with a Beckman Acta III recording spectrophotometer at 550 nm in 3 ml of a medium that consisted of 20 *mM* potassium phosphate (pH 7.5), 2 *mM* KCN, and 0.15 *mM* cytochrome c. Mitochondrial samples (1 mg protein) were incubated in the reaction medium for 2 min to lyse the membranes, and then the phthalate ester was added and the sample was incubated for an additional 2 min. Reduction of cytochrome c was initiated by the addition of succinate (2 *mM* final concentration).

Reagent grade DBP was obtained from Fisher Scientific Co. Technical grade DEHP was obtained from the Hatco Chemical Division of W. R. Grace and Co. MEHP and MBP were synthesized by refluxing phthalic anhydride and 2-ethylhexanol or *n*-butanol in toluene and were then extracted as described by Albro et al. (*13*). Stock solutions of each of the phthalate esters were made in 95% ethanol.

Results

Energy-Dependent K$^+$ Uptake

In intact, coupled mitochondria, oxidation of added substrate or the hydrolysis of adenosine-5'-triphosphate (ATP) generates a potential across the inner membrane composed of a pH gradient plus an electrical gradient (the outside is positive with respect to the inside). The active transport of cations across the inner mitochondrial membrane is driven by the membrane electrical potential. K$^+$ is naturally impermeable to the inner mitochondrial membrane; however, the lipid-soluble ionophore, valinomycin, has a polar interior that delocalizes the cation charge so that the K$^+$ valinomycin complex can diffuse across the membrane. The energy-linked accumulation of K$^+$ in response to a charge gradient results in osmotic water flow which leads to mitochondrial swelling. The swelling results in greater light penetration through the mitochondrial suspension, and can be followed by monitoring changes in light absorption (*14*). We examined the effects of phthalate esters on energy-dependent K$^+$ uptake driven by the oxidation of two respiratory chain substrates, succinate and ascorbate-TMPD, and by the hydrolysis of ATP. We reasoned that, if there is a substrate specific effect, then K$^+$ transport would be affected differently with the different energy sources; and if the phthalate esters induce a nonspecific effect, then the energy-linked K$^+$ transport would be affected similarly with the three energy sources.

Table 1 presents a comparison of the effects of phthalate esters on energy-linked K$^+$ uptake in isolated rat liver mitochondria. These effects were determined by monitoring changes in absorbance at 540 nm. In the control, there is a rapid decrease in absorbance upon addition of valinomycin. A typical trace of change in absorbance at 540 nm of rat liver mitochondria due to succinate oxidation-induced K$^+$ uptake is shown in Figure 1. The maximum amplitude of swelling is reached about 30 sec after the addition of valinomycin. The mitochondria undergo a series of swellings and contractions until the oscillations become damped, largely due to a loss in synchrony and to membrane deterioration occurring during the swelling and contraction cycles (*11*). These volume changes do not occur if substrate oxidation is inhibited or if the membrane potential is collapsed by addition of an uncoupler.

In the presence of 1.0 *mM* MBP, swelling initiated with each of the three energy sources is slower than in the control and there is a loss of synchrony of swelling and contraction. Similar observations

Table 1. Effect of phthalate esters on energy-linked K$^+$ uptake in isolated rat liver mitochondria.[a]

	Energy source		
Condition	Succinate	Ascorbate-TMPD	ATP
MBP			
0.01*mM*	+ + + +	+ + + +	+ + +
1.0*mM*	+ + +	+ + +	+ +
DBP			
0.010*mM*	+ + + +	+ + + +	+ +
0.025*mM*	+ + +	+ + +	+
0.1*mM*	–	–	
1.0*mM*	–	–	
MEHP			
0.1*mM*	+ + +	+ + +	+
1.0*mM*	–	+	
DEHP			
0.1*mM*	+ + + +	+ + + +	+ + +
1.0*mM*	+ + + +	+ + + +	+ +

[a] Energy-linked swelling; (+ + + +) equivalent to the control; (+ + +) amplitude of swelling is equivalent to the control, but there is a loss of oscillations; (+ +) substantially decreased amplitude of swelling with no oscillations; (+) very slight uncoupler sensitive swelling; (–) no detectable energy-linked swelling.

have been made with uncouplers at low concentrations and are, therefore, indicative of a reduced energy supply (*14*). Reduction of the concentration of MEHP to 0.1 *mM* results in absorbance changes that are nearly identical to that of the control. In the presence of 1.0 *mM* MEHP, swelling is abolished with succinate as the energy source; however, there is slight uncoupler-sensitive swelling with ascorbate–TMPD or ATP. Therefore, in addition to a common effect with each of the three energy sources, there is an additional effect of MEHP on succinate oxidation-linked swelling. Reduction of the concentration of MEHP to 0.1 *mM* resulted in increased active transport activity in each case. In the presence of 1.0 *mM* DEHP, the swelling traces with succinate and ascorbate–TMPD are similar to the control, indicating that there is no apparent deleterious effect. A slight alteration in ATP-supported K^+ uptake occurs at 1.0 *mM* DEHP. Since DEHP did not affect succinate or ascorbate-TMPD induced swelling, it is possible that the adenine nucleotide translocator or the ATPase is partially sensitive to DEHP. In the presence of 1.0 or 0.1 *mM* DBP, energy-linked K^+ transport initiated by oxidation of succinate or ascorbate–TMPD or by hydrolysis of ATP is totally abolished. In each case, energy-linked swelling (sensitive to uncoupler) is markedly improved when the concentration of DBP is reduced to 0.025 *mM*. At 0.010 *mM* DBP, the swelling traces are nearly identical to the control traces.

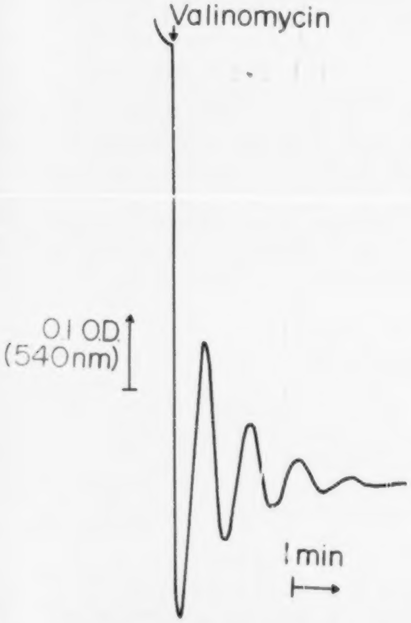

FIGURE 1. Oscillation of rat liver mitochondria due to K^+ transport linked to succinate oxidation.

Respiratory Control

Table 2 demonstrates the effects of phthalate esters on mitochondrial respiration with succinate as the oxidizable substrate. In coupled mitochondria, the proton electrochemical gradient that is generated during substrate oxidation suppresses electron transport to oxygen. The addition of ADP stimulates electron flow and oxygen consumption since this energized state is being utilized, in this case for ATP synthesis. The respiratory control ratio (RCR) is a measure of the dependence of the respiratory rate on the presence of ADP (state 3) divided by the rate after the expenditure of ADP (state 4). ADP stimulation represents the incre-

Table 2. Effect of phthalate esters on mitochondrial respiration.

Conditi	RCR[a]	State 4 rate (% of control)	State 3 rate (% of control)	ADP stimulation (% of control)
Control	3.7	100[b]	100[c]	100[d]
MBP				
0.1*mM*	3.1	123	96	87
1.0*mM*	2.7	144	101	84
DBP				
0.1*mM*	1.4	285	89	33
1.0*mM*	1.1	297	82	9
MEHP				
0.1*mM*	1.6	167	71	34
1.0*mM*	–	109	22	0
DEHP				
0.1*mM*	3.6	103	96	93
1.0*mM*	3.6	101	98	96

[a] Respiratory control ratio.
[b] 9.7 nmole oxygen/min-mg protein.
[c] 35.9 nmole oxygen/min-mg protein.
[d] State 3 rate – State 4 rate; 26.2 nmole oxygen/min-mg protein.

ment in the respiratory rate after the addition of ADP. The respiratory control ratio, which is indicative of coupling between the oxidation and phosphorylation processes, is regarded as a good index of the integrity of mitochondrial structure and function. Generally, an increase in state 4 respiration leading to a loss of respiratory control is indicative of uncoupling by dissipation of the proton electrochemical gradient. Classical uncouplers (e.g., 2,4-dinitrophenol) act as proton ionophores promoting proton equilibration across the inner mitochondrial membrane. Inhibited respiratory rates are largely attributed to inhibition of substrate oxidation or ADP utilization. The changes in respiratory control due to the presence of the phthalate esters are similar to those reported by Takahashi (*6*). The greatest changes were observed (Table 2) with DBP and MEHP. There was a moderate effect with MBP, and there was essentially no change in the presence of DEHP.

The decrease in respiratory control due to MBP can be accounted for by the increase in state 4 respiration. DBP is an effective uncoupler of oxidative phosphorylation at concentrations of 1.0 and 0.1 *mM*. The effect is largely due to stimulation of state 4 respiration. The reduced rates in the presence of ADP (state 3 rate) indicate that there is also some inhibition of succinate oxidation or ADP utilization. As a result of these effects, there is nearly total loss in stimulation of respiration upon addition of ADP, i.e., there is essentially no phosphorylation of ADP. MEHP also promotes a loss of respiratory control. At 0.1 *mM* MEHP there is stimulation of state 4 respiration, and both 0.1 *mM* and 1.0 *mM* MEHP markedly decrease state 3 respiration rates. The latter effects are probably due to inhibition of succinate oxidation since the respiratory rates are not stimulated by the uncoupler, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), but are stimulated upon subsequent addition of ascorbate + TMPD (results not shown). Since ascorbate + TMPD donate electrons into the respiratory chain at cytochrome c, then inhibition of succinate oxidation by MEHP must occur between succinate dehydrogenase and cytochrome c. Thus, MEHP appears to act as an uncoupler of oxidative phosphorylation and an inhibitor of succinate oxidation. The combination of these effects limits or eliminates ATP synthesis, as indicated by the loss of ADP stimulation of state 4 respiration. DEHP had no apparent effect on mitochondrial respiration.

Succinate Cytochrome c Reductase Activity

Succinate cytochrome c reductase is a partial reaction of the electron transport chain, in which succinate serves as the electron donor and exces cytochrome c is provided as the electron accepto (*12*). Potassium cyanide is added to block electro flow from cytochrome c to oxygen. The rate o cytochrome c reduction is measured spectrophoto metrically at 550 nm. Since the catalytic site o succinate dehydrogenase is located on the inne surface of the inner mitochondrial membrane, th reaction was measured with osmotically lysed mito chondria in order to eliminate the carrier-mediate transport of succinate as the potentially rate-limitin step. MBP and DEHP had no effect on the activity while DBP showed significant inhibition (Table 3) MEHP was the most potent inhibitor of the phthalat esters that were tested. In each case that inhibitio was observed, the rate of cytochrome c reductio was increased maximally upon addition of NADH Since both NADH and succinate cytochrome c reduc tase activities include the cytochrome b-c_1 region o the electron transport chain, then the inhibite rates shown in Table 3 can be attributed to inhibi tion of succinate dehydrogenase activity.

Discussion

The present studies show that phthalate ester affect isolated rat liver mitochondria by uncouplin energy-linked processes and by inhibiting succinat dehydrogenase activity. The phthalate esters tha were examined vary in their potency in affectin these processes. MBP had a similar effect on th energy-linked K^+ uptake for the three differen energy sources employed. It also stimulated succinate state 4 respiration rates. Based on these effect MBP can be considered to be a weak mitochondria uncoupler. Classical uncouplers, such as FCCP totally uncouple oxidative phosphorylation at micromolar concentrations (*15*). DBP was an inhibito

Table 3. Effect of phthalate esters on succinate cytochrome reductase activity of lysed mitochondria.

Condition	Succinate cytochrome c reductase activity, % of control
Control	100[a]
MBP	
0.1*mM*	106
1.0*mM*	95
DBP	
0.1*mM*	65
1.0*mM*	56
MEHP	
0.1*mM*	67
1.0*mM*	23
DEHP	
0.1*mM*	96
1.0*mM*	98

[a] 118 nmole/min-mg protein.

of succinate dehydrogenase activity and was the most potent uncoupler tested. It interfered with K$^+$ uptake induced by all three energy sources; it stimulated by nearly threefold the succinate state 4 respiration rates; and it led to a nearly total loss of respiratory control. DBP is not a weak acid, as are the classical mitochondrial uncouplers that increase the passive permeability of the inner mitochondrial membrane to protons. Therefore, DBP probably uncouples energy-linked processes by partitioning in the mitochondrial membrane causing molecular conformational changes that render the membrane permeable to protons and other small ions. In support of this conclusion, Inouye et al. (*7*) found that exposure of isolated mitochondria to DBP results in release of intramitochondrial K$^+$ in the absence of valinomycin. In addition, Ohyama (*16*) reported that DBP stimulates the latent ATPase activity of whole mitochondria at concentrations that also induced nonenergy-linked mitochondrial swelling. These observations are consistent without contention that DBP alters the permeability properties of the inner mitochondrial membrane.

MEHP is also an uncoupler of mitochondrial energy-linked reactions. It impaired the energy-linked K$^+$ uptake initiated with three different energy sources, and it stimulated succinate state 4 respiration rates. There was total loss of respiratory control in the presence of 1.0 *mM* MEHP. Of the phthalate esters that were tested, MEHP was the most potent inhibitor of succinate dehydrogenase activity. We are currently investigating the nature of that inhibition. DEHP had no effect on liver mitochondrial activities other than weak inhibition of ATP-supported K$^+$ uptake.

Although these studies were conducted on isolated mitochondria, it is possible that the permeability properties of other cellular membranes are similarly altered by phthalate esters. The net effect of energy uncoupling and inhibition of substrate oxidation is a diminished chemical energy supply and altered regulation of ion transport in cells exposed to such compounds. These changes could modify energy-dependent cytoplasmic activities and the activities of ion-dependent enzymes. MEHP is a mitochondrial energy uncoupler and an inhibitor of succinate dehydrogenase activity. Since MEHP is a metabolite of DEHP, then it may play an important role in the hepatotoxic effects associated with exposure to DEHP.

It is interesting to note some of the similarities in effects of DEHP and clofibrate in rodents. Oral administration of either compound induces proliferation of peroxisomes (*3, 17, 18*), reduces plasma cholesterol and triglyceride levels (*17, 19*) and results in the development of hepatocellular carcinomas (*2, 20*). In addition, clofibrate ethyl ester has been shown to stimulate succinate oxidation rates of isolated mouse liver mitochondria, while the pharmacologically active metabolite formed by hydrolysis of the ester, sodium clofibrate, inhibits succinate oxidation (*21*). MEHP possesses an ester and a free carboxyl group, and depending on its concentration and the presence of ADP, MEHP stimulates or inhibits succinate oxidation rates of rat liver mitochondria. It is tempting to speculate that some of the effects of MEHP and clofibrate on rodent hepatocytes are a consequence of a primary effect on the mitochondria. The mitochondrial responses should occur before the other changes induced by these compounds are expressed. Therefore, we suggest that peroxisome proliferation may be a result of a cellular response to inhibition of mitochondrial succinate oxidation, and hypolipidemia may in part reflect decreased lipid biosynthesis due to reduced mitochondrial ATP synthesis or increased oxidation resulting from loss of respiratory control.

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Environmental Health Perspectives
Vol. 45, pp. 57-64, 1982

Hepatic Effects of a Phthalate Ester Plasticizer Leached from Poly(vinyl Chloride) Blood Bags Following Transfusion

by Sherwin V. Kevy* and May S. Jacobson*

The effects of di(2-ethylhexyl) phthalate (DEHP) on hepatic function and histology were evaluated in the rhesus monkey undergoing platelet and plasma transfusion. The average cumulative amount infused in one year is comparable to that received by patients who undergo chronic transfusion. Abnormalities in hepatic scan and BSP kinetics persisted for up to 26 months after transfusion, as did histologic abnormalities. Patients undergoing maintenance hemodialysis receive a yearly dose of DEHP which is 10-20 times that which produced hepatotoxicity in the transfused rhesus.

The plasticizer di(2-ethylhexyl) phthalate (DEHP) is incorporated into poly(vinyl chloride) (PVC) medical devices to produce flexibility. PVC medical devices such as blood bags and indwelling intravenous catheters contain up to 40% by weight of DEHP. This material is leached from PVC by blood and lipoprotein-containing solutions (*1*). This solubilization is time- and temperature-dependent (*1*). Previous studies have demonstrated DEHP in the tissues of multitransfused patients and in neonates whose only exposure was indwelling umbilical catheters (*2, 3*).

Jacobson and Kevy (*4*) have demonstrated that DEHP levels of 5 mg/dl, the concentration found in whole blood stored for 21 days at 4°C and in platelets stored at 22°C for 24 hr, significantly inhibits the growth of human diploid fibroblasts. As a consequence of this finding, rhesus monkeys were used in a chronic transfusion study in which PVC blood bags were compared with those made of polyethylene.

The rhesus monkey was chosen as the animal model, since there is marked species difference in the metabolism of DEHP. Their baseline serum protein analysis and agarose electrophoresis pattern are normal in terms of human values except for the orosomucoid level which is slightly lower. In addition they behave similarly to both children and adults as far as compartmentalization analysis of bromosulfophthalein (BSP) kinetics and hepatic ^{99m}Technetium-labeled sulfur colloid scanning techniques.

These animals were transfused with either platelet-rich or platelet-poor plasma in a manner identical to that utilized in clinical medicine. This is an extremely critical point, because virtually all the toxicology literature relative to DEHP is based upon rodent exposure in a manner which in no way mimics the way the human is exposed via transfusion of blood products or renal dialysis.

Materials and Methods

Experimental Groups

Immature rhesus monkeys approximately six months old and weighing less than 5 lb were divided into the four following groups.

PVC Platelet-Rich Plasma (PRP) Transfused. Three rhesus monkeys were transfused weekly for one year with 15 ml of PRP stored for 48 hr at 22°C, and two monkeys received PRP stored at 4°C.

PVC Platelet-Poor Plasma (Plasma) Transfused. Two monkeys were transfused biweekly for 6 months with 15 ml of plasma stored at 22°C for 5 days. Even though this is no longer standard blood bank practice, plasma was stored at room temperature to maximize the leaching of DEHP.

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Nontransfused Controls. Three monkeys lived in the same environment and underwent the same test procedures as the experimental rhesus.

Transfused Controls. Two monkeys were transfused weekly for 6 months with 15 ml of PRP stored for 48 hr at 22°C in polyethylene blood bags.

An aliquot of each transfusion was quantitated for DEHP content.

Preparation of Platelet-Rich Plasma

Adult donor monkeys were plasmapheresed biweekly to obtain platelet-rich plasma. Blood, 100 ml, was collected into a 150 ml PVC or polyethylene blood pack aseptically filled with 16 ml ACD anticoagulant. The blood was centrifuged in a SBV No. 1 International centrifuge at 500 *g* at room temperature for 10 min, and the platelet-rich plasma was aseptically transferred into a second 150 ml PVC or polyethylene transfer pack. These packs were then stored at 4 or 22°C for 48 hr. The average platelet count in each bag was 150,000/mm^3. The red cells were returned to the donor monkeys.

Preparation of Plasma

Adult donor monkeys were plasmapheresed into PVC blood packs as detailed above, except that the whole blood was centrifuged at 2520 *g* for 10 min in a RC-3 Sorvall centrifuge. The plasma was transferred into a PVC transfer pack and stored at 22°C for up to 5 days. Each unit of plasma was tested and found to be platelet free.

DEHP Quantitation

Levels of DEHP in blood products, blood and tissue were determined by gas chromatography with the use of our modification of the method of Jaeger (*4*). The sensitivity of the assay is 0.1 ppm (parts per million).

DEHP Dosage

The amount of DEHP received by an experimental animal was calculated as the number of milliliters of stored blood component received times its DEHP concentration. The total amount of DEHP was determined by a summation of the results of each transfusion.

Hepatic Scintillation Scanning Techniques

^{99m}Technetium-labeled sulfur colloid provides stable count rates, is short-lived, and has favorable radiation dosimetry (*5*). Each rhesus monkey received ^{99m}Tc at dose of 0.015 mCi/kg body weight. Within 45 min after injection, the abdomen was scanned. Peak counts were recorded over the substance of the liver and spleen for 3 min and repeated five times. The hepatosplenic ratio was then determined from the mean counts over the liver and the spleen.

Sulfobromophthalein (BSP) Transport

Sulfobromophthalein (BSP) transport was evaluated by the method of Quarfordt and associates (*6*). Each study was performed after at least a 12-hr fast. BSP was injected intravenously in a dose of 5 mg/kg of body weight. Sequential blood samples were obtained from the arm opposite that used for injection. Zero time for the study was set at the midpoint of the injection, which took an average of 30 sec to complete. Blood samples were obtained prior to injection for BSP recovery studies, then at 3-min intervals for 30 min, and thereafter at 15-min intervals for up to 2 hr. After the final blood sample, urine was collected for BSP recovery.

The plasma disappearance curve following a single injection of BSP was analyzed by a computer technique for determining compartmental flow. The method computes the hepatic removal rate (HRR), hepatic to plasma reflux rate (H-PRR), the biliary secretory rate (BSR), and the urinary excretion rate (UER) by means of the simulation analysis and modeling technique of Berman and associates, with the use of the SAAM digital computer program on an IBM 370-5165 computer (*7*). These rates are expressed as the fractional rate (i.e., percent transported) per minute.

The model was verified in the rhesus monkey by measurement of the disappearance of dye from the plasma and quantitation of the concentration of dye excreted in the bile and urine. The bile samples were obtained by cannulation of the common bile duct. The experimentally observed values were plotted along with the computer-calculated values and the curves were superimposed. An injected dose was established which would not saturate the transport processes. Data obtained by these methods were compared to previously reported studies by us (*8*) in normal children and adults and children with cystic fibrosis (CF).

Histopathology

Multiple surgical liver biopsies were performed on all transfused and nontransfused monkeys. In order to insure adequate sampling for all studies, a wedge biopsy was obtained on each occasion. Tissues were rapidly fixed in formalin and embedded in paraffin. Sections were stained by hematoxylin-eosin, periodic acid-Schiff, periodic acid-Schiff-alcohol and Sudan Black. All preparations were examined in blinded fashion by four observers.

Results

DEHP Dosage

At the start of this study ten consecutive patients were identified who predictably would be multi-transfused. During the course of one year, each unit of red cells, whole blood, plasma and platelets was quantitated for DEHP. The amount of DEHP the patients received ranged from 56 to 1500 mg or 2.1 to 27.5 mg/kg. During their one-year period of transfusion the group infused with platelet-rich plasma stored at 22°C received 76.8-96.0 mg of DEHP or 24-30 mg/kg, those transfused with platelet-rich plasma stored at 4°C received 20.5–27.0 mg or 6.6-8.7 mg/kg and those infused with plasma 108 mg or 33 mg/kg.

Assessment of Liver Function

^{99m}Tc Sulfur Colloid Liver-Spleen Scans. Severe or moderate impairment of hepatic perfusion and/or infiltration are detected by irregular distribution of tracer within the liver. Milder forms of parenchymal disease can be quantitated by determination of the distribution ratio of radiocolloid between liver and spleen. Each animal served as its own control. All subsequent determinations of the hepatosplenic ratio were compared to the baseline ratio obtained prior to transfusion. Liver/spleen activity ratios were calculated assuming the spleen to be one. A ratio was considered abnormal if there was at least a 30% decrease from the baseline level.

The results of the scanning studies in all monkeys are shown in Table 1. All rhesus transfused with platelets stored in PVC had a significant decrease in ratio throughout the period of transfusion therapy. One rhesus in each of the platelet transfusion groups were sacrificed at varying intervals during this study to determine the DEHP organ content. Three of the five surviving monkeys who were transfused from PVC-DEHP blood bags demonstrated abnormal scans for up to the 26 months post-transfusion that they were followed during the course of this study.

There were no changes in the hepatosplenic ratios in the monkeys transfused with platelets stored in polyethylene or in the nontransfused controls.

BSP Transport. The plasma disappearance curves for BSP and the data from the two-compartment model of Quarfordt provide a very sensitive indicator of subclinical liver disease. We have demonstrated that the transport of BSP in the normal monkey and the normal human being are identical. The normal immature rhesus monkey and the normal child have a single exponential curve, whereas the normal adult rhesus monkey and the normal adult human have a biexponential curve.

The change in the plasma BSP disappearance curve is proportional to the degree of liver involvement. A child with cyctic fibrosis (CF) who has no evidence of hepatomegaly and normal liver function demonstrates a single exponential disappearance curve identical to that of a normal child. The child with CF who has hepatomegaly and biopsy documented liver pathology demonstrates a biexponential disappearance curve, even though SGOT, SGPT, alkaline phosphatase, bilirubin and standard BSP tests are normal. The development of a biexponential disappearance curve in a child or immature subhuman primate, therefore, serves as a sensitive marker of hepatic dysfunction and is considered abnormal. One can also distinguish in the adult the difference between a normal biexponential disappearance curve and that produced by liver disease (*8*).

Table 1. Rhesus liver/spleen activity ratios following ^{99m} Tc sulfur colloid infusion.

	Abnormal/normal					
		Transfusion interval		Interval after transfusion		
Rhesus group	Baseline	6 mo.	12 mo.	5 mo.	14 mo.	26 mo.
Nontransfused	0/3	0/3	0/3	0/3	0/3	0/3
22°C – PRP – PVC	0/3	3/3	3/3	2/3	2/3	1/2
4°C – PRP – PVC	0/2	2/2	2/2	1/1	1/1	1/1
Plasma – PVC	0/2	1/2	–	1/2	1/2	1/2
PRP – Polyethylene	0/2	0/2	0/2	0/2	0/2	0/2

The results of the compartmental analysis of BSP kinetics in the transfused and nontransfused immature rhesus monkeys are shown in Table 2. A BSP analysis was considered abnormal if the disappearance curve was biexponential. The monkeys transfused with platelets stored in polyethylene and the nontransfused controls did not exhibit any abnormalities throughout the course of this study. Four of the five monkeys transfused with platelets stored in PVC exhibited abnormal disappearance curves following one year of platelet transfusions. This persisted in three of the four remaining rhesus monkeys 14 months after transfusion and in one rhesus 26 months following cessation of transfusions. Both rhesus transfused with plasma stored in PVC had abnormal BSP disappearance curves immediately following the six months of transfusion. These reverted to normal within five months following the cessation of transfusions.

Liver Histopathology. A biopsy was considered abnormal when at least two of the following findings were present: (1) foci of parenchymal necrosis (aggregates of chronic inflammatory cells and Kupffer cells in the parenchymal with degenerating hepatocytes); (2) vacuolization of Kupffer cells; (3) chronic inflammatory cell infiltrates; and (4) prominence and/or increase in the number of Kupffer cells and the presence of binucleate cells. At no time during this study were any histologic abnormalities identified in liver biopsies of nontransfused or polyethylene-transfused control monkeys.

All of the animals transfused with platelets or plasma stored in PVC demonstrated histologic abnormalities throughout the follow-up period.

Figure 1 demonstrates finding obtained in biopsy 24 months following cessation of transfusion in a monkey who had received platelets stored at 4°C. Figure 2 demonstrates the persisting histologic findings obtained at necropsy 32 months after the cessation of transfusions in one of the monkeys transfused with platelet-rich plasma stored at 22°C.

Histologic changes at this time were characterized by aggregates of inflammatory cells, hepatocyte degeneration, and multinucleated and binucleated giant cells. These findings persisted espite the fact that no DEHP was detected in live tissue at the time of necropsy.

Tissue Levels of DEHP

One of the inherent difficulties of liver bi sies is that only small specimens can be obtained Tissue samples obtained at biopsy averaged 225 g wet weight (range 100–400 mg). The limit of se itivity of our analysis for DEHP by G.C. is 0.1 pp . Any samples with DEHP contents of 1.4 ppn to 1.5 ppm, levels well above background, were onsidered to have only a trace amount. Table 3 ts the DEHP content of liver tissue obtained oth at biopsy and necropsy.

As shown in Table 3. the initial biopsies all the PVC transfused rhesus contained significant nounts of DEHP. The nontransfused and polyet ylene-transfused monkeys had no detectable DEH P. The five month follow-up samples contained con centrations similar to those initially measured with the exception of one PRP-transfused monkey and the two plasma-transfused animals that only had trace amounts of DEHP. At the 14 month follow-up biopsy of the PRP animals, three had only trace amounts of DEHP, and one had no detectable level. Since these samples were in the lower range of the sensitivity of our assay for DEHP, we sacrificed one of the animals (rhesus "G"). The tissue samples utilized ranged from 2.5 to 4.0 g wet weight. Significant levels of DEHP were found in the liver, testis, heart and omental fat. The total residual organ level, excluding fat, was less than one percent of the dose administered.

DEHP was not detected in liver samples o tained at necropsy 32 months after transfusion, lespite the persistence of abnormal histologic finding Unfortunately, none of the samples were analyze for the monoester (MEHP).

Hemodialysis Studies

Since the rate of leaching of DEHP from VC is dependent on the temperature and lipid co tent of

Table 2. Rhesus BSP kinetics.

Rhesus group	Abnormal/normal					
		Transfusion interval		Interval after transfusion		
	Baseline	6 mo.	12 mo.	5 mo.	14 mo.	26 mo.
Nontransfused	0/3	0/3	0/3	0/3	0/3	[illegible]3
22°C – PRP – PVC	0/3	3/3	3/3	2/3	2/3	[illegible]2
4°C – PRP – PVC	0/2	0/2	1/2	1/1	1/1	[illegible]1
Plasma – PVC	0/2	2/2	–	0/2	0/2	[illegible]2
PRP – Polyethylene	0/2	0/2	0/2	0/2	0/2	[illegible]2

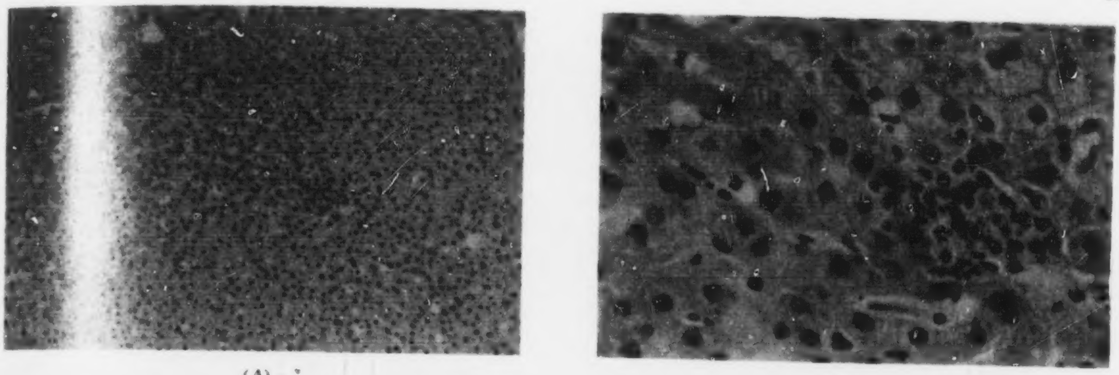
(*A*) (*B*)

FIGURE 1. Biopsy 26 months after cessation of transfusions: (*A*) rhesus D received platelet-rich plasma stored for 48 hr at 22°C in PVC (100 ×); (*B*) rhesus D; areas shown demonstrate binucleate cells, hepatocyte necrosis and degenerating hepatocytes (400 ×).

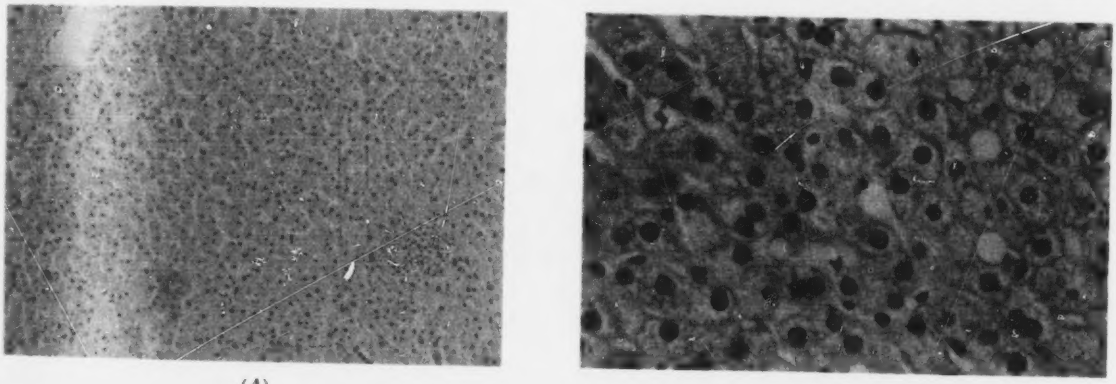
(*A*) (*B*)

FIGURE 2. Necropsy 32 months after cessation of transfusions: (*A*) 100 ×; (*B*) 400 ×. Areas shown demonstrate aggregates of inflammatory cells, hepatocyte necrosis and binucleate cells. DEHP content: none detected.

Table 3. DEHP content in liver of biopsied monkeys.[a]

			DEHP content, μg/g wet weight of liver				
				Interval after transfusion			
Animals	Procedure	Total DEHP infused, mg	Initial biopsy	5 mo.	14 mo.	26 mo.	32 mo. (necropsy)
B	N	0	ND[c]	ND	ND	ND	ND
C	N	0	ND	ND	ND	ND	ND
M		0	ND	ND	ND	ND	ND
D	(PVC)–PRP	74.80	3.0	Trace	ND	6.7	ND
F	(PVC)–PRP	96.00	5.0	3.8	Trace	ND	ND
G	(PVC)–PRP	78.72	10.0	9.2	0.7 (Necropsy)	Deceased	
E	(PVC)–PRP	27.00	1.0	1.8	Deceased		
H	(PVC)–PRP	20.52	1.5	3.1	Trace	Deceased	
S	(PVC)Plasma	96.00	10.1	Trace	Trace	ND (Necropsy)	
T	(PVC)Plasma	108.00	18.1	Trace	18.7	Trace (Necropsy)	
L	(PE)–PRP	0	ND	ND	ND	ND	
P	(PE)–PRP	0	ND	ND	ND	ND	

[a] Initial biopsy performed on PRP animals after 1 year of duration; initial biopsy performed on plasma animals after 6 months of transfusion.

[b] NT = not transfused.

[c] ND = none detected.

the media, dialysis patients who characteristically have elevated serum lipids are at increased risk as compared to chronically transfused patients.

Blood samples for DEHP analysis were obtained before dialysis and from venous and arterial lines at 1, 2, 3 and 4 hr and immediately after dialysis. Samples of dialysate fluid were obtained from both the inflow and outflow sites during the indicated time intervals. The estimated amount of DEHP received by a patient was calculated from the concentration per milliliter of venous blood times the flow rate multiplied by the duration of dialysis.

As shown in Table 4, DEHP was not demonstrated in the predialysis venous blood samples. All patients showed DEHP in the venous blood but none in the arterial blood during hemodialysis. The estimated total amount of DEHP delivered to each patient during a single dialysis varied from 32 mg to 90 mg. In one year the estimated total amount of DEHP received by a patient would range from 120 mg/kg to 335 mg/kg, a dose 10 to 20 times that which produced liver abnormalities in the rhesus monkey.

Intensive Plasma Exchange

Intensive plasma exchanges were performed with an IBM Blood Cell Separator, 5% albumin being used as replacement fluid. Blood flow rate varied between 50 and 80 cc/min. Blood samples were obtained before plasma exchange and at 30, 60, 90 min and at the conclusion of the exchange.

Three patients requiring maintenance plasma exchange were studied (Table 5). Patient 3 had a total lipid level of 800 mg-% (cholesterol 24 ng-%, phospholipids 225 mg-%, triglycerides 335 g-%). The estimated total amount delivered to each atient during a single two-plasma volume plasma ex ange, varied from 3.3 mg in patients with norm l lipid levels to 11.6 mg in the patient with elevated lasma lipids. During a single year this patient rec ves an estimated 290 mg of DEHP, or 3.3 mg/ . The patient has undergone plasma exchange r two years.

Discussion

The methods used in previously reported tudies call for a more critical assessment of an toxic hazard created by the use of phthalate este s, as it is impossible to establish the significance in man of *in vitro* and *in vivo* studies with an insoluble substance. The amount of DEHP leached into platelets or plasma stored in PVC blood bags is based on the situation that exists in clinical medicine. The rhesus monkey was chosen as an animal model because its red cell survival, platelet preservation characteristics, serum protein pattern and DEHP metabolism are similar to those of the human.

Hepatic disease is often clinically occult due to the remarkable hepatic functional reserve and regenerative capability. The technetium hepatic scintillation scan is a satisfactory method for visually recording abnormal function of diminished perfusion. The latter effect results in impaired hepatic extraction of radioactivity which gives rise to a nonuniform image (5). In mild forms of parenchy-

Table 4. DEHP plasma concentration during hemodialysis.

	DEHP plasma concentration, μg/ml					
		Venous DEHP				
Patient	Prevenous	1 hr	2 hr	3 hr	3.5 hr	Estimate total DEHP rece ed, mg
1	ND[a]	3.0	3.8	5.0	4.9	90
2	ND	1.8	4.0	3.5		32
3	ND	2.9	2.5	3.2		57

[a]ND = none detected.

Table 5. DEHP plasma concentration during continuous flow: intensive plasma exchange.[a]

		Plasma DEHP, μg/ml			Estimated total DEHP recei ed, mg	
Patient	Prevenous	30 min	60 min	90 min	Per procedure	Per y r
1	ND[b]	2.9	2.6	2.3	3.5	87.
2	ND	1.7	1.8	2.0	3.3	
3	ND	5.7	4.1	3.2	11.6	290.

[a]IBM cell separator.
[b]ND = none detected.

mal disease, not manifested by irregular distribution of the liver, changes can be quantitated by determining the relative distribution of radiocolloid between liver and spleen (*9*).

Sulfobromophthalein metabolism and transport may be evaluated in several ways. The standard method determines the retention of BSP at a specific interval, usually 45 min after a single injection (*10*). A more sensitive technique is the utilization of a two-compartmental model for the analysis of BSP kinetics. This was first described in the dog, subsequently validated in the human by Quarfordt et al. and in the rhesus monkey by ourselves. The observation that the normal BSP disappearance curve in the child and immature primate is a single exponential and only changes to a biexponential in the presence of liver disease or with age is of significance to the present study (*8*, *11*). At the time this study was completed, none of the rhesus had shed any of their deciduous teeth, nor had they reached sexual maturity. Their average weight was 4.54 kg. One of the monkeys who was part of the study to validate the BSP model had multiple determinations during a three-year period and converted from a single exponential to a biexponential. This occurred at approximately 5.5 years and at a weight of 10.45 kg. The biexponential curves noted in this study and those seen in children with mild liver disease differs from those produced by chronic injury in which the initial slope flattens due to the decrease in the hepatic removal rate (*8*).

Even though the number of animals in each of the groups is too small for valid statistical analysis, a trend is observed in the PVC transfused monkeys. In the present study, prolonged abnormalities in hepatic scan and BSP kinetics occurred following DEHP infusion without changes in standard liver function tests. Six of seven animals had abnormal ^{99m}Tc scan ratios, and six of seven demonstrated abnormal BSP compartmental flow upon completion of transfusion therapy. These findings have persisted for up to 14 months following therapy in three of the four monkeys given PRP and one of the two monkeys transfused with plasma. Two the the three remaining monkeys given PRP continued to have abnormal ^{99m}Tc scan ratios 26 months following the cessation of transfusion therapy. The results of the serial liver biopsies in the PVC transfused monkeys were quite dramatic and all were abnormal except for the initial biopsy on rhesus "G" which was normal. Disturbances of hepatic architecture, the presence of round-cell infiltration, and multinucleated giant cells persisted throughout the follow-up period. The histologic abnormalities have been reported in apparently normal subhuman primates. Although none of the animals in this study had pretreatment biopsies, all seven PVC-transfused rhesus monkeys and none of the six control rhesus had abnormal histology. The probability of this being a chance occurrence is highly unlikely.

Although monkeys transfused with plasma stored in PVC received equivalent doses of DEHP to those in the PVC-PRP group, their duration of exposure was significantly shorter. This may account for the fact that histological changes in liver biopsy were milder and that their liver function as reflected in the kinetics of BSP transport returned to normal during the follow-up period.

The results of DEHP quantitation on the serial liver biopsies of the PVC transfused monkeys shows a decrease in the residual DEHP content after the cessation of transfusion. Although we have not as yet established the metabolic pathway of DEHP in the rhesus monkey, this finding supports the work of Daniel et al. who have demonstrated in the rat and Albro et al. in the African Green monkey that the DEHP is metabolized in part to mono(2-ethylhexyl) phthalate (MEHP) (*12*, *13*). This metabolite is a potent inhibitor of liver dehydrogenases, and theoretically may have greater potential for hepatotoxicity than the parent compound.

Based upon a comparison of the abnormalities observed in the platelet-rich plasma and plasma transfused rhesus, a larger dosage over a prolonged time period appears to be more toxic. This is clinically significant to patients undergoing hemodialysis who yearly are exposed to far greater amounts of DEHP than that which produced hepatic toxicity in the rhesus. These patients are more prone to liver disease which cannot be attributed to the hepatitis B surface antigen or non-A, non-B hepatitis.

PVC medical devices have a high degree of reliability and fabrication technology. While the histological and functional abnormalities induced by PVC plasticized with DEHP in the ranges used in this study are mild, they were produced at dose levels observed in transfusion therapy and at lower levels than those that are observed in hemodialysis.

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Environmental Health Perspectives
Vol. 45, pp. 65-70, 1982

Teratogenicity of Di(2-ethylhexyl) Phthalate (DEHP) and Di-*n*-butyl Phthalate (DBP) in Mice

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Di(2-ethylhexyl) phthalate (DEHP) and di-*n*-butyl phthalate (DBP) were mixed with diet at graded levels of 0.05, 0.1, 0.2, 0.4 and 1.0 wt-% and given to pregnant ICR mice throughout gestation. Maternal weight gain was suppressed and fetal resorptions increased at 0.2, 0.4 and 1.0% levels of DEHP and 1.0% level of DBP. All the implanted ova died early in rats fed 0.4 and 1.0% levels of DEHP. External malformations increased significantly by 0.2% DEHP, and 1.0% DBP showed borderline significance. The major malformations in treated groups were neural tube defects (exencephaly and myeloschisis), suggesting that the phthalic acid esters (PAEs) affect neural tube closure in developing embryos. Treatment with the compounds caused intrauterine growth retardation and delayed ossification with an apparently dose-related response pattern. These results indicate that a high dose of DEHP and DBP might be embryotoxic and teratogenic in mice. The maximum nonembryotoxic doses of PAEs in mice were more than 2000 times the estimated level of human intake through the food chain. Thus it is assumed that the current "normal" exposure level of PAEs does not pose an imminent threat to human fetal development.

Introduction

Phthalic acid esters (PAEs) have been reported to leach into plasma from blood storage bags (*1*) and hemodialysis units (*2*). These compounds have also been found in a variety of materials in the environment, including foodstuffs (*3*). Thus, pregnant women might be at risk for unintentional exposure to minute amounts of PAEs derived from medical devices and diet.

In recent years, many studies have been carried out on the possible health hazards of PAEs, including the effects on reproduction and fetal development, and they have been extensively reviewed by several investigators (*4-6*). However, there is still a paucity or inconsistency of teratological information of PAEs. Singh et al. (*7*) injected several kinds of PAEs intraperitoneally into rats on days 5, 10 and 15 of gestation. Fetal resorptions and gross abnormalities increased on injection of 10 ml/kg di(2-ethylhexyl) phthalate (DEHP) and 1.02 ml/kg di-*n*-butyl phthalate (DBP). Skeletal abnormalities and hematomas were observed at 0.610 and 0.305 ml/kg levels of DBP. Nikonorow et al. (*8*) administered 0.34 and 1.70 g/kg/day DEHP and 0.12 and 0.60 g/kg/day DBP orally to rats during pregnancy and observed increased resorptions and decreased fetal weight, but no gross malformations. In a mouse study, consecutive oral administration of 10 mg/day DEHP and 10 and 50 mg/day DBP to pregnant females of ICR and ddY strains induced abnormalities in their offspring, including renal cysts and absence of tail (*9*). In this short report, however, the data were not compared with appropriate controls. Single oral administration of DEHP to pregnant mice of a random strain on day 7 of gestation resulted in decreased fetal weight at a dose level of 0.05 ml/kg and increased number of dead and malformed fetuses at 0.1 ml/kg (*10*). Teratogenicity in chicks has been reported by Haberman and his co-workers (*11-13*). Mutagenic and antifertility effects of DEHP have been shown by Singh et al. (*14*) by dominant lethal test in mice.

The present study was undertaken to ascertain the effects of orally administered DEHP and DBP

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on embryonic development and to determine the maximum nonembryotoxic dose in mice for extrapolation to the human. In the present experiment, the test compounds were given to the animals with diet throughout the whole gestation period, because they are ingested by people mainly through the food chain.

Materials and Methods

DEHP and DBP manufactured by Tokyo Kasei Industrial Co., Ltd. (Tokyo) were provided by the Ministry of Health and Welfare, Japan. They were individually mixed with the basal diet) OA-2 (CLEA Japan, Inc., Takatsuki) at graded levels of 0.05, 0.1, 0.2, 0.4 and 1.0 wt-%; the highest dose level was estimated to be near the maximum nontoxic dose for mature female ICR mice by a preparatory subchronic study for 3 weeks.

Four-week-old male and female mice of ICR-JCL strain were purchased from CLEA Japan, Inc. and reared in our laboratory with laboratory chow (OA-2) and tap water *ad libitum*. The room temperature was kept at 22 ± 2°C and the relative humidity at 55 ± 5%. The daily light cycle was 12 hr light and 12 hr dark. Each female mouse at 8-16 weeks of age was placed overnight with a male of proven fertility. The day on which a vaginal plug was found was taken as day zero of pregnancy. The females with vaginal plugs were randomly distributed into ten test groups and one control group. The females in the test groups were fed pellets containing DEHP or DBP at the graded levels mentioned above from day zero until the day of sacrifice. The control animals were fed the basal diet only. During the gestation period, the females were weighed daily, and food and water consumptions recorded daily.

On day 18 of pregnancy, the females were sacrificed by cervical dislocation. The uteri were removed, and implantations, resorptions and dead fetuses were recorded. The live fetuses were weighed, sexed and inspected for gross external abnormalities. About half of the fetuses from each litter, randomly selected, were eviscerated, fixed in 95% ethanol, cleared in 1% KOH, and stained with alizarin red S for detection of skeletal abnormalities. The remaining fetuses were fixed in Bouin solution for several days and examined for internal soft tissue anomalies by microdissection under a dissecting microscope.

The data concerning maternal weight and food and water consumption were analyzed by Student's *t* test for comparing means between groups. The data relating fetuses were analyzed in the following way in order to minimize biases resulting from the litter effect (*15*). The percentages or means for a group were computed by first obtaining the percentage or mean for each litter and then calculating the average of these percentages or means. The distribution of a percentage or a mean for treated litters was compared with the control distribution by use of the Wilcoxon rank sum test (*16*).

Results

Maternal Food, Water and PAE Intake

The average food intake by the treated females ranged from 6.1 to 7.4 g/day, and these did not differ significantly from the amount taken by the control mice (Table 1). No dose-related trends were noted in food consumption. The average daily doses of DEHP calculated from food intake and body weight were 2200, 830, 410, 190 and 70 mg/kg for dose levels of 1.0, 0.4, 0.2, 0.1 and 0.05%, respectively. The corresponding doses of DBP were 2100, 660, 370, 180 and 80 mg/kg for the respective dose levels. The daily water intake was not significantly different in treated and control groups.

Table 1. Effects of DEHP and DBP on pregnant mice.

Phthalate	Phthalate dose, %	Number of dams	Average food intake, g/day	PAE dosage, mg/kg/day	Average maternal weight, g (mean ± SE)	
					Day 0	Day 1
DEHP	1.0	12	7.2	2,200	30.2 ± 0.4	37.4 ± 2[a]
DEHP	0.4	7	7.2	830	31.4 ± 0.9	38.1 ± 1[a]
DEHP	0.2	24	6.9	410	29.0 ± 0.8	42.6 ± 2[b]
DEHP	0.1	9	7.4	190	29.2 ± 0.6	49.9 ± 3
DEHP	0.05	8	6.1	70	31.6 ± 0.8	56.6 ± 1
DBP	1.0	15	7.1	2,100	30.4 ± 0.4	39.7 ± 6[a]
DBP	0.4	9	6.9	660	29.9 ± 0.3	53.5 ± .9
DBP	0.2	21	7.1	370	30.1 ± 0.7	52.6 ± .5
DBP	0.1	8	7.3	180	29.5 ± 0.4	52.2 ± .8
DBP	0.05	7	6.9	80	29.3 ± 0.3	56.3 ± .5
Control	—	8	6.2	–	29.2 ± 0.4	52.6 ± .1

[a] Significantly different from control mice ($p < 0.01$).
[b] Significantly different from control mice ($0.01 < p < 0.05$).

Effects on Dams

No mortality or behavioral abnormalities were observed in the pregnant mice throughout the experimental period. However, the maternal weight was significantly depressed during the later gestation period in the groups given 1.0, 0.4 and 0.2% DEHP and 1.0% DBP (Table 1). Such weight suppression of the mothers could be accounted for by the increased early resorptions in these groups. At autopsy, no gross pathological changes were noted in maternal major organs.

Effects on Fetal Viability and Weight

There was no significant difference in the mean numbers of corpora lutea and implantations between the treated and control groups (Table 2). Fetal mortality increased in a dose-related manner, and it was significantly higher in 1.0, 0.4 and 0.2% DEHP and 1.0% DBP groups. No viable term fetuses were obtained at the two highest doses of DEHP. Most of the fetal deaths were very early resorptions.

The average weight of viable fetuses showed a dose-related decrease in the treated groups, and that in 0.2% DEHP group and 0.4% DBP group (male only) was significantly lower than in controls (Table 2). Out of the 15 pregnant females which were given 1.0% DBP, only three fetuses from two dams survived to term. Their weights were far lower than those of controls.

External Anomalies

Several kinds of malformations were observed in the treated groups (Table 3). The incidence of malformed fetuses in 0.2% DEHP group was significantly higher than in the control group ($p < 0.05$). The difference between 1.0% DBP and control groups was at the borderline level of significance ($p \approx 0.05$). The most common malformations were neural tube defects (exencephaly and myeloschisis), and

Table 2. Effects of DEHP and DBP on fetal viability and development.

Phthalate	Phthalate dose, %	Number of litters	Number of implants (means ± SE)	Resorptions and dead fetuses, %	Fetal weight, g (mean ± SE)	
					Male	Female
DEHP	1.0	12	12.6 ± 0.5	100.0[a]	–	–
DEHP	0.4	7	15.0 ± 1.2	100.0[a]	–	–
DEHP	0.2	24	11.1 ± 0.9	67.8[a]	1.12 ± 0.07[a]	1.16 ± 0.04[b]
DEHP	0.1	9	12.6 ± 2.1	31.0[c]	1.28 ± 0.08	1.21 ± 0.07
DEHP	0.05	8	13.3 ± 1.7	7.5	1.32 ± 0.07	1.29 ± 0.06
DBP	1.0	15	12.1 ± 0.5	98.4[a]	1.03, 1.24	0.80
DBP	0.4	9	12.0 ± 0.8	11.4	1.10 ± 0.18[b]	1.06 ± 0.20
DBP	0.2	21	12.4 ± 0.8	22.3	1.31 ± 0.07	1.24 ± 0.06
DBP	0.1	8	12.0 ± 2.9	11.2	1.29 ± 0.10	1.22 ± 0.08
DBP	0.05	7	12.7 ± 3.1	3.7	1.31 ± 0.04	1.29 ± 0.03
Control	—	8	10.8 ± 1.9	5.0	1.41 ± 0.07	1.35 ± 0.07

[a] Significantly different from control mice ($p < 0.01$).
[b] Significantly different from control mice ($0.01 < p < 0.05$).
[c] Difference from controls is at the borderline level of significance ($p \approx 0.05$).

Table 3. Malformations in mouse fetuses from dams treated with DEHP or DBP.

Phthalate	Phthalate dose, %	Number (%) of malformed fetuses	Type of malformation and (no. of cases)[c]
DEHP	1.0	—	—
DEHP	0.4	—	—
DEHP	0.2	14 (25.8)[a]	Ex (3), Ex + OE (2), Ex + TA (1), My (1), My + GS + CF (1), My + OE + GE (1), GS + TA (1), TA (4)
DEHP	0.1	3 (5.3)	Ex (2), OE (1)
DEHP	0.05	0 (0.0)	—
DBP	1.0	2 (75.0)[b]	Ex (2)
DBP	0.4	0 (0.0)	—
DBP	0.2	1 (0.5)	OE (1)
DBP	0.1	0 (0.0)	—
DBP	0.05	0 (0.0)	—
Control	—	0 (0.0)	—

[a] Significantly different from control mice ($p < 0.05$).
[b] Difference from controls is at the borderline level of significance ($p \approx 0.05$).
[c] Type of malformation: CF club foots; Ex, exencephaly; GE, generalized edema; GS, gastroschisis; My, myeloschisis; OE, open eyelids; TA, tail anomaly.

Table 4. Effects of DEHP and DBP on skeletal development of mouse fetuses.

Phthalate	Phthalate dose, %	Number of fetuses examined	Skeletal malformations	Variations (%)		Number of ossified coccygia (mean ± SE)
				Lumbar rib	Deficient ossification of sternebrae	
DEHP	1.0	0	–	–	–	–
DEHP	0.4	0	–	–	–	–
DEHP	0.2	40	0	18.7	8.3	6.5 ± 0.
DEHP	0.1	35	0	20.7	14.7	9.2 ± 0.
DEHP	0.05	31	0	7.6	0.0	7.3 ± 0.
DBP	1.0	0	0	–	–	–
DBP	0.4	42	0	36.9	0.0	2.6 ± 0.
DBP	0.2	65	0	26.3	0.0	6.0 ± 0.
DBP	0.1	38	0	17.2	0.0	4.5 ± 0.
DBP	0.05	41	0	23.8	6.3	5.1 ± 0.
Control	—	0	0	13.3	0.0	9.4 ± 0.

[a]Significantly different from control mice ($p < 0.05$).

Table 5. Reported teratogenicity/embryotoxicity of DEHP in rodents.

Species (strain)	Route of administration	Period of administration	Dose	Principal findings	Authors
Mouse (ICR)	PO	Throughout gestation	190-2200 mg/kg/day	Neural tube defects, tail anomalies, decreased fetal weight, delayed ossification, resorptions	Present study
Mouse (ICR, ddY)	PO	Throughout gestation	10 mg/day	Renal cysts	Onda et al. (*9*)
Mouse (ddY-Slc × CBA)	PO	Day 7	0.05 ml/kg 0.1 ml/kg	Decreased fetal weight Fetal deaths, skeletal abnormalities	Nakamura et al.
Rat (Wistar)	PO	Throughout gestation	0.34, 1.70 g/kg/day	Resorptions, decreased fetal weight, decreased placental weight	Nikonorow et al.
Rat (Sprague-Dawley)	PO	Days 3, 6, 9	2.4 ml/kg/day	Decreased implantations	Peters and Cook
Rat (Sprague-Dawley)	IP	Days 5, 10, 15	10 ml/kg/day	Malformations, resorptions, decreased fetal weight	Singh et al. (*7*)

these anomalies were occasionally encountered also in dead fetuses. Other anomalies consisted of gastroschisis, absence or shortness of tail, club foot, open eyelids and generalized edema.

Skeletal and Visceral Anomalies

As shown in Table 4, extra lumbar ribs and deficient ossification of sternebrae increased in treated groups, although the difference from the controls was not statistically significant. Ossification was significantly retarded in treated groups except 0.1% DEHP group. Delayed ossification in those groups was probably related to the general underdevelopment of the fetuses. No internal anomalies were observed by microdissection.

Discussion

The results of the present experiment indicate that DEHP and DBP can affect fetal development when they are taken orally during pregnancy. Although there appears to be no adverse effect on implantation, a dose-related response was obser for fetal mortality and fetal body weight. It also shown that DEHP and DBP provoke t teratogenicity in mice at high dosages.

In the two previous experiments, in which DE and/or DBP was given orally to pregnant mice *10*), cysts of the kidney, absence of tail (*9*) skeletal abnormalities (*10*) were noted, but no ne tube defects were observed. On the contrary, r cysts were not found in the present study by car visceral examination. It is also interesting to that a certain difference in embryotoxicity has b shown between mice and rats, and between (PO) and parenteral (IP) administration (Tabl and 6). At present, there is no clear explanation such inconsistency between experiments. Altho recent metabolic studies have shown that PAEs metabolized in rats after oral administration (*17* and IV injection (*20*), comparative data are insufficient in the mouse and human. It seems pos that the metabolism of PAEs is not the same betw species and between strains. Thus compara aspects of PAE metabolism and potential em

Table 6. Reported teratogenicity/embryotoxicity of DBP in rodents.

Species (strain)	Route of administration	Period of administration	Dose	Principal findings	Authors
Mouse (ICR)	PO	Throughout gestation	660, 2100 mg/kg/day	Neural tube defects, resorptions, decreased fetal weight, delayed ossification	Present study
Mouse (ICR, ddY)	PO	Throughout gestation	10, 50 mg/day	Renal cysts, absence of tail	Onda et al. (*9*)
Rat (Wistar)	PO	Throughout gestation	0.12, 0.60 g/kg/day	Resorptions, decreased fetal weight	Nikonorow et al. (*8*)
Rat (Sprague-Dawley)	IP	Days 5, 10, 15	0.61, 0.305 ml/kg/day	Decreased fetal weight, skeletal abnormalities	Singh et al. (*7*)
			1.02 ml/kg/day	Decreased fetal weight, resorptions	

otoxicity of the metabolites should be ascertained. Pertinent animal models in terms of the pharmacokinetic similarity to humans are needed.

From the findings of our experiment, it seems reasonable to assume that the maximum nonembryotoxic doses of orally administered PAEs in mice would be at least 70 mg/kg/day for DEHP and 370 mg/kg/day for DBP. On the other hand, the current human intake of PAEs from the diet has been estimated to be 0.03 mg/kg/day at maximum in the case of the Japanese (*10*, *21*). Thus the maximum nonembryotoxic doses in mice are more than 2000 times the current human exposure level reported so far. For this reason, it seems unlikely that ingestion of PAEs under the current environmental level would pose an imminent threat to human early development. The following facts also favor this assumption. First, pharmacokinetic studies show that DEHP is metabolized and eliminated rapidly in mice (*22*, *23*), rats (*18*, *24*) and humans (*1*, *25*). Second, Singh et al. (*26*) found that in rats the levels of phthalate diesters in a whole fetus did not exceed the level in the maternal blood. In the human, DEHP and DBP in cord blood were at about the same levels as in the maternal blood (*27*). Thus, no fetal accumulation of PAEs is likely to occur. Third, Garvin et al. (*28*) and Lewandowski et al. (*29*) injected IV the plasma-soluble extracts of poly(vinyl chloride) (PVC) to pregnant rats and found no evidence of teratogenic or embryotoxic effects. In one of the experiments (*29*), the highest dose of the extract was equivalent to 5.3 mg DEHP/kg/day, which is similar to the amount predicted to be received by a 60-kg human undergoing an exchange transfusion of 21-day-old blood.

Although placental transfer of PAEs has been studied in rats (*26*), there is no information concerning the accumulation of PAEs in human conceptuses. Since there is little expectation of human *in vivo* study, actual determination of the PAE levels in human embryonic and fetal tissues and comparison of those with the levels in experimental animals with recognizable embryotoxicity would enable us to assess how safe the current environmental level is to human conceptuses. Such determination of fetal tissue levels could be done by utilizing the tissues from induced human abortuses (*30*).

From the discussion above, it can be concluded that the current "normal" dietary exposure level of PAEs may be harmless to human development. However, the problem of high level exposure in the human has not yet been solved. Epidemiological studies are needed regarding the reproductive outcome in populations under high level exposure of PAEs, e.g., among woman workers in the PVC and car industries.

The authors are gratetul to Dr. M. J. Chou for his technical assistance. This work was supported by the Japanese Ministry of Health and Welfare's Contract Fund of Food Sanitation Research in 1975 entitled "Safety Evaluation of Chemical Substances Contained in Plastic Apparatus and Package."

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Environmental Health Perspectives
Vol. 45, pp. 71-75, 1982

Teratogenicity/Fetotoxicity of DEHP in Mice

by I. Tomita,* Y. Nakamura,* Y. Yagi* and K. Tutikawa†

The embryonic/fetotoxic effects of DEHP on pregnant mice (ddY-Slc ♀ × CBA ♂) were studied. DEHP was administered orally in dosages of 0.05 ml/kg to 30.0 mg/kg on day 6, 7, 8, 9 or 10 of gestation. A single administration of DEHP over 0.1 ml/kg (1/300 of LD_{50}) on day 7 of gestation decreased the numbers and the body weight of living fetuses, whereas no significant changes in the numbers of living fetuses (with no gross and skeletal abnormalities) were observed compared with those of the control group, when 0.05 ml/kg (1/600 of LD_{50}) of DEHP was administered. The fetotoxicity (fetal death) was dose dependent. The LD_{50} and the nonfetolethal maximum dosage of DEHP in its single, oral administration was 592 mg/kg and 64 mg/kg, respectively. The latter value is much higher than an estimated DEHP intake from commercial foodstuffs in men, which is approximately 0.03 mg/kg/day.

Introduction

The deleterious effects of di(2-ethylhexyl) phthalate (DEHP) on developing embryos and/or fetuses in animals have been reported by a number of investigators (*1-14*).

The present reports are the summaries of our studies (*15, 16*) carried out to determine the embryo toxicity and fetotoxicity of DEHP on ddY-Slc(SPF) (♀) × CBA(SPF)(♂) mice. DEHP was administered orally once during the period of pregnancy. The dosages administered were from 1/30 to 1/1 of LD_{50} (high-dose study) and from 1/600 to 1/30 of LD_{50} (low-dose study).

Through these studies, the nontoxic maximum dose of DEHP in mice was determined, and steps were taken to assess the risk of DEHP for human reproduction.

Experimental

Virgin female mice of the ddY-Slc (SPF) strain (Shizuoka Agricultural Cooperative Association for Laboratory Animals), weighing 27-28 g (8-9 weeks old), were housed with one male mouse of CBA strain (National Institute of Genetics). The atmosphere was maintained at 25 ± 2°C and 50 ± 5% RH. Laboratory diet (Funahashi Farm Co.) and tap water were provided *ad libitum*. DEHP (purity over 99%) was obtained from Tokyo Kasei Co. Ltd. MEHP was synthesized according to the method of Kenyon (*17*) and was more than 99% pure as determined by ECD-GLC analysis. The experimental procedure is detailed in our previous paper (*16*).

Results

Figure 1 shows the body weight changes of pregnant mice. The increase in body weight of the mice was suppressed when 10 ml/kg of DEHP was given PO once on day 7, 8, 9, or 10 of gestation. The suppression was especially clear when DEHP was administered on day 7 or 8 of gestation. A decrease in the body weight of mice, however, was not observed when a low dosage of DEHP (0.05 ml and 0.1 ml/kg) was given, even on day 7 of gestation (see inset in Fig. 1).

Table I summarizes the effects of DEHP on the fetuses of mice. The study consisted of four groups: an untreated control group, two DEHP-treated groups (high-and low-dose groups) and an ethylurethane-treated group (positive control). As shown in Table 1, the number of implantations per pregnant mouse was not significantly different among the control and DEHP-treated groups, but the numbers of embryos which suffered an early or late death varied greatly, depending on the day and the

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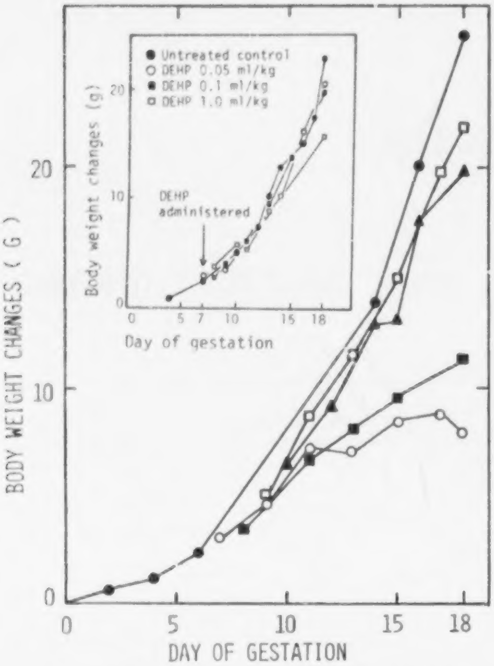

FIGURE 1. Effects of di(2-ethylhexyl) phthalate (DEHP) on the body weight changes of pregnant mice. DEHP was given PO in one dose of 10 ml/kg on (○) the 7th, (■) 8th, (□) 9th, or (Δ) 10th day of gestation; (●) untreated control.

amount of DEHP administered. A high incidence of dead embryos was found in mice which had received DEHP on day 7 or 8 of gestation, whereas a low incidence of dead embryos was found in mice which had received DEHP on days 6, 9, or 10. It must be noted that a higher incidence of early and late deaths among embryos occurred with the higher and lower dosages of DEHP administration, respectively. There was a high rate of gross and skeletal abnormalities among the two groups of mice which had received 2.5 or 7.5 ml of DEHP/kg on days 7 or 8 of gestation, respectively. No abnormality was observed in the other two groups of mice receiving 0.05 or 0.1 ml DEHP/kg although the average body weight of viable fetuses was significantly lower ($p < 0.01$) than that of the control group.

The details of the gross and skeletal malformation in mouse fetuses due to DEHP administration are shown in Table 2. Exencephaly, open eyelid, club foot and bent or no tail were often found in the malformed fetuses, while thoracic lumbar, sacral and caudal vertebrae were often found in an abnormal shape. There was no case of a cleft palate.

Figure 2 shows the correlation between the death and abnormalities (gross and skeletal) of fetuses versus the dosage of DEHP administered on day 7 of gestation. From a straight line ($y = 51.9 \log x + 61.6$, where y = % of fetal death, x = ml/kg of DEHP administered) obtained with 5 log dosage of DEHP, the fetal LD_{50} was determined to be 592 mg/kg on assuming the specific gravity of DEHP to be 0.986. The dosage producing 2% of fetal deaths, i.e., the same mortality rate as found in the control group, was about 64 mg/kg. The exact values of the ineffective maximum dosage for gross and skeletal abnormalities could not be determined because of limited data, but these values are probably less than 0.80 ml/kg (789 mg/kg) and 0.68 ml/kg (670 mg/kg), respectively, as judged from their dose-response curve.

Our trial for the safety evaluation of DEHP with respect to embryo/fetal toxicity is shown in Figure 3. As was demonstrated and stated above, the maximum nonteratogenic (both gross and skeletal) dose and median fetolethal dose (fetal LD_{50}) in mice are approximately the same, around 600 mg/kg, while the maximum nonfetolethal dose in mice is about 64 mg/kg. In humans, approximately 4 mg/kg would be a possible maximum intake of DEHP by blood transfusion or hemodialysis (*18*). The level (unconditional) of the acceptable daily intake for humans (ADI) is about 1 mg/kg/day (*19*).

The maximum intake of DEHP from foodstuffs with which we are most concerned would not exceed 0.03 mg/kg/day, assuming a daily food intake as 1.5 kg/50 kg adult and an average content of DEHP in our foodstuffs as 1 ppm (*20*).

A safety margin of approximately 21-fold thus exists between the nonfetolethal dose of DEHP and the maximum amount of DEHP from foodstuffs, if a 100-fold margin is simply set to extrapolate the data from mice to humans.

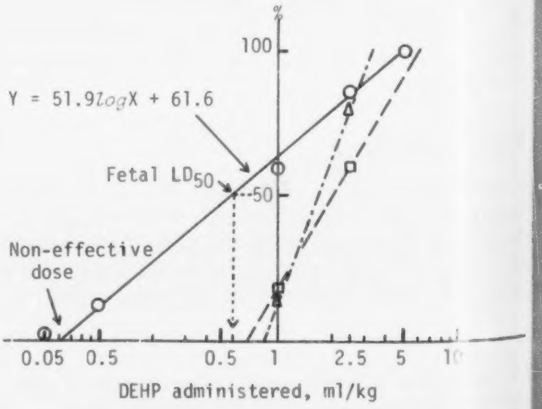

FIGURE 2. Fetotoxicity of DEHP in mice: (○) fetal death; (Δ) gross abnormalities; (□) skeletal abnormalities.

Table 1. Effects of DEHP on mouse fetuses.

Group	Day of gestation	Dose administered, ml/kg[a,b]	Number of implantations (no. pregnant mice)	Number of dead embryos (% live fetuses)		Number of living embryos (% live fetuses)[c]	Numbers of abnormalities[d]		Average body weight of fetuses, g
				Early death	Late death		Gross	Skeletal	
(High dose) untreated control	–	–	56(5)	0	0	56(100)	0	0	1.41 ± 0.06
DEHP (high dose)	6	2.5(1/12)	60(6)	1(1.7)	7(11.7)	52(86.7)	1(1.9)	3(5.8)	1.31 ± 0.12[e]
	7	1.0(1/30)	54(6)	13(24.1)	19(35.2)	22(40.7)	3(13.6)	4(18.2)	1.20 ± 0.09[e]
		2.5(1/12)	36(5)	21(58.3)	10(27.8)	5(13.9)	4(80.0)	3(60.0)	1.17 ± 0.18[e]
		5.0(1/6)	43(4)	27(62.8)	16(37.2)	0			
		10.0(1/3)	59(5)	44(74.6)	15(25.4)	0			
	8	7.5(1/4)	66(6)	8(12.1)	29(43.9)	29(43.9)	19(65.5)	24(82.8)	1.07 ± 0.07[e]
		10.0(1/3)	83(8)	15(18.1)	53(63.9)	15(18.1)	7(46.7)	5(33.3)	1.23 ± 0.14[e]
	9	7.5(1/4)	30(3)	2(6.7)	1(3.3)	27(90.0)	1(3.7)	0	1.28 ± 0.08[e]
		10.0(1/3)	48(5)	2(4.2)	2(4.2)	44(91.7)	9(20.5)	0	1.32 ± 0.09[e]
		30.0(1/1)	48(5)	2(4.2)	16(33.3)	30(62.5)	9(30.0)	5(16.7)	1.08 ± 0.12[e]
	10	10.0(1/3)	65(7)	2(3.1)	1(1.5)	62(95.4)	0	0	1.37 ± 0.13[f]
		30.0(1/1)	63(7)	3(4.8)	3(4.8)	57(90.5)	4(6.5)	0	1.28 ± 0.16[e]
(Low dose) untreated control	–	–	301(31)	2(0.7)	4(1.3)	259(38.0)	0	0	1.39 ± 0.06
DEHP (low dose)	7	0.05(1/600)	200(22)	0	5(2.5)	195(97.5)	0	0	1.31 ± 0.11[e]
		0.1(1/300)	98(11)	2(2.0)	9(9.2)	87(88.8)	0	0	1.27 ± 0.13[e]
		1.0(1/30)	94(10)	1(1.1)	55(58.5)	38(40.4)	3(7.9)	7(18.4)	1.22 ± 0.10[e]
Ethylurethane (IP)	7	1.5	34(4)	1(2.9)	5(14.7)	28(82.4)	4(14.3)	4(14.3)	1.08 ± 0.22
	8	1.5	44(5)	3(6.8)	0	41(93.2)	1(2.4)	1(2.4)	1.20 ± 0.10
	9	1.5	41(4)	1(2.4)	25(61.0)	15(36.6)	13(86.7)	15(100)	0.85 ± 0.12
	10	1.5	22(3)	0	0	22(100)	20(90.9)	22(100)	0.87 ± 0.06

[a] Oral administration.
[b] Numbers in parentheses denote fraction of acute LD_{50} (30 ml/kg).
[c] Numbers in parentheses indicate percent of live fetuses based on total number of implantations.
[d] Numbers in parentheses indicate percent abnormalities based on total numbers of live fetuses.
[e] Significantly different from untreated control at 99% level ($p < 0.01$).
[f] Significantly different from untreated control at 95% level ($p < 0.05$).

Table 2. Incidence of gross and skeletal malformations in the fetus of mouse after di(2-ethylhexyl) phthalate (DEHP) administration at various days of gestation.

	Untreated control	Day 6	Day 7		Day 8		Day 9			Day 10	
		2.5 (ml/kg)	1.0 (ml/kg)	2.5 ml/kg	7.5 (ml/kg)	10.0 ml/kg	7.5 (ml/kg)	10.0 ml/kg	30.0 (ml/kg)	10.0 ml/kg	30.0 (ml/kg)
No. fetuses examined	56	60	54	36	66	83	30	48	48	63	65
No. live fetuses	(56)	(52)	(22)	(5)	(29)	(15)	(27)	(44)	(30)	(62)	(57)
No. gross abnormalities	0	1	3	4	19	7	1	9	9	0	4
(%)[a]		(1.9)	(13.6)	(80.0)	(65.5)	(46.7)	(3.7)	(20.5)	(30.0)		(6.5)
Exencephaly	0	0	1	0	7	2	0	0	0	0	0
Open eyes	0	0	1	0	5	7	0	8	3	0	0
Abdominal hernia	0	0	0	0	1	0	0	0	0	0	0
Club foot	0	0	1	1	5	3	1	1	2	0	0
Poly-dactyly	0	0	0	0	1	0	0	0	0	0	1
Bent tail bone	0	1	1	4	5	4	0	0	0	0	5
Subcutaneous bleeding	0	0	0	0	0	0	0	0	4	0	0
No. skeletal abnormalities	0	3	4	3	24	5	0	0	5	0	0
(%)[a]		(5.8)	(18.2)	(60.0)	(82.8)	(33.3)			(16.7)		
Skull vertebrae	0	0	1	0	7	2	0	0	0	0	0
Cervical vertebrae	0	2	0	0	0	0	0	0	0	0	0
Thoracic vertebrae	0	2	4	2	21	5	0	0	1	0	0
Lumbar vertebrae	0	0	1	3	8	4	0	0	3	0	0
Sacral vertebrae	0	0	1	1	6	4	0	0	1	0	0
Caudal vertebrae	0	1	1	5	3	0	0	0	0	0	0
Ribs	0	1	4	2	20	5	0	0	0	0	0

[a] Percent gross or skeletal malformations based on total number of live fetuses examined.

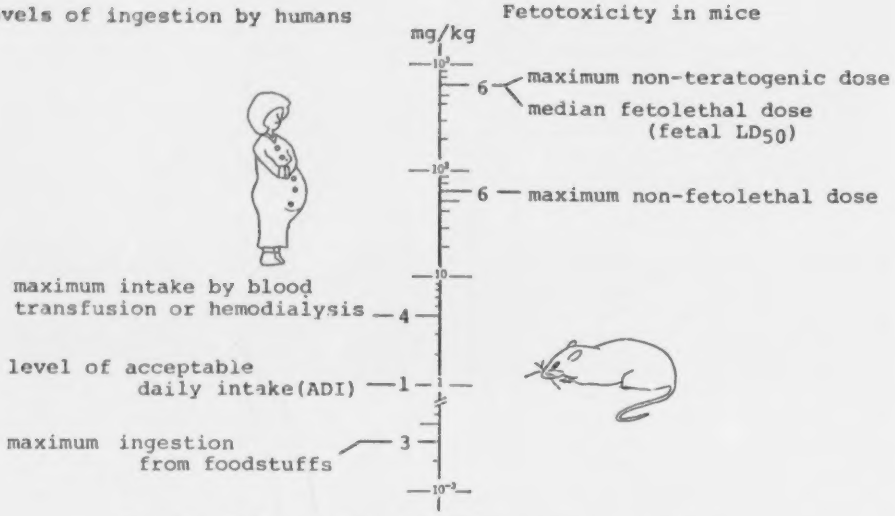

FIGURE 3. Safety evaluation of DEHP in respect to fetotoxicity.

Discussion

Consistent results have not always been obtained in the reproductive/fetotoxic studies of DEHP, due mainly to differences in the dosage of DEHP, the duration and the route of administration. The animal species employed in the experiments is another crucial factor in determining the toxic effects. The effects of DEHP on reproductive function or fetal toxicity in several animal species have been performed by several investigators and are well reviewed (*9*, *12*). The toxic effects of DEHP on the mouse fetuses which have been demonstrated in recent examinations (*7*, *15*, *16*) should be added. It must be also added that the metabolic fate of DEHP in mice seems to be somewhat similar to that in man when solely the metabolic formation of MEHP is considered (*21*). As MEHP is known to be a principal—and possibly the active—metabolite of DEHP (*12*, *16*, *22*), the similarity between mice and humans may have some advantage in extrapolating the mouse data to men.

As shown in Figure 3, the nonfetolethal maximum dose of DEHP in mice by a single oral administration is estimated to be about 64 mg/kg. This value is approximately 2100 times that (0.03 mg/kg) which is a conceivable level of DEHP intake from commercial foodstuffs. Judging exclusively from the above experimental results and estimation, it seems to be unlikely that DEHP will produce any immediate threat to human reproduction, though the conclusive assessment of DEHP should await further study.

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Environmental Health Perspectives
Vol. 45, pp. 77-84, 1982

Testicular Effects of Phthalate Esters

by S. D. Gangolli*

The testicular effects produced by di(2-ethylhexyl) phthalate (DEHP) in the rat, characterized by a decrease in the relative organ weight and histological changes in the seminiferous tubules, can also be produced by di-*n*-butyl, di-*n*-pentyl and di-*n*-hexyl phthalates. The corresponding monoesters of these compounds, formed *in vivo* as a result of the action of nonspecific esterases in the intestinal mucosa and other tissues, were equally effective in inducing testicular damage. Phthalate-induced testicular injury was accompanied by a decrease in the zinc content in the gonads and in increased urinary excretion of this element.

Exposure of preparations of rat seminiferous tubule cells in culture to monophthalates capable of producing testicular injury resulted in a dose-related detachment of germinal cells from Sertoli cells in a manner similar to the effect seen in the intact animal. This *in vitro* system may find application in the elucidation of the toxic mechanisms involved in phthalate-induced testicular injury and in screening compounds likely to act in a manner similar to phthalates.

This paper presents a brief review of some of the earlier work on phthalate-induced testicular injury followed by the results of studies conducted at BIBRA.

The first reported finding of phthalate-induced testicular injury in experimental animals was by Shaffer et al. (*1*) in 1945. The oral administration of di(2-ethylhexyl) phthalate (DEHP) at dietary concentrations of 0.075, 0.75, 1.5 and 5.0% to rats for 90 days resulted in tubular atrophy and testicular degeneration resembling senile changes at the two top dose levels. Subsequently, Harris et al. (*2*) found occasional incidence of tubular atrophy in rats fed DEHP at 0.5% in the diet for periods of 3 or 24 months.

Other reported instances of phthalate treatment leading to testicular effects in experimental animals were by Calley et al. (*3*), who found that the daily intraperitoneal administration (250 mg/kg body weight) of DEHP or di(methoxyethyl) phthalate in mice for [illegible] weeks significantly reduced the relative testicular weight. Furthermore, these two compounds administered IP at half or two-thirds the LD_{50} dose levels produced an antifertility effect in male mice (*4*).

A 90-day feeding study of DEHP in the rat conducted at BIBRA (*5*) showed that the administration of the compound at 0.2, 1.0 and 2.0% in the diet resulted not only in a dose-related increase in relative liver weight in the treated animals but also a decrease in the relative testes weight of rats on 1.0 and 2.0% DEHP (Table 1). There was histological evidence of testicular injury, and additionally, "castration" cells in the pituitary at all treatment levels. The histopathological changes in the testes from DEHP-treated animals (Fig. 1) were characterized by a marked reduction in the diameter of seminiferous tubules, the germinal epithelium consisted only of Sertoli cells, spermatogonia and a few spermatocytes, and there was cessation of spermatogenesis. The interstitial tissue and Leydig cells appeared normal. The testicular atrophy produced by DEHP at the 2% dietary level occurred within two weeks of treatment. In the first series of experiments on the ability of phthalate esters to produce testicular injury in the rat, a target organ study of two weeks' duration showed that, whereas diethyl phthalate had no discernible adverse effects on the testes, di-*n*-butyl phthalate (DBP) produced testicular atrophy, possibly more severe than that produced by DEHP.

Studies by Cater et al. (*6*) on DBP showed that the oral administration of DBP at daily dose levels of 500 and 1000 mg/kg resulted in a significant reduction in the relative testes weight within 6 days and 4 days, respectively (Table 2). The corresponding monoester (MBP), the major urinary metabolite of DBP (*7*), was found to be even more effective in reducing the testes weight. The histopathology of the testicular lesion produced by DBP or MBP was similar to that following DEHP treatment.

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Table 1. Summary of findings on the testicular effects produced by DEHP in a 90-day feeding study in the at.

DEHP treatment				Histological findings: incid ce of	
Dietary level, % (w/w)	Mean daily intake, mg/kg	No. of rats	Relative testes weight, g/100 g body weight	Testicular injury[a]	"Castra n" cells in pi tary
0	0	15	0.60	0	
0.2	150	15	0.61	4 (+)	
1.0	750	15	0.41[b]	12 (+ + +)	
2.0	1500	15	0.23[b]	15 (+ + +)	

[a] Severity of testicular damage: (+) slight; (+ +) moderate; (+ + +) severe.
[b] Statistical significance $p < 0.001$.

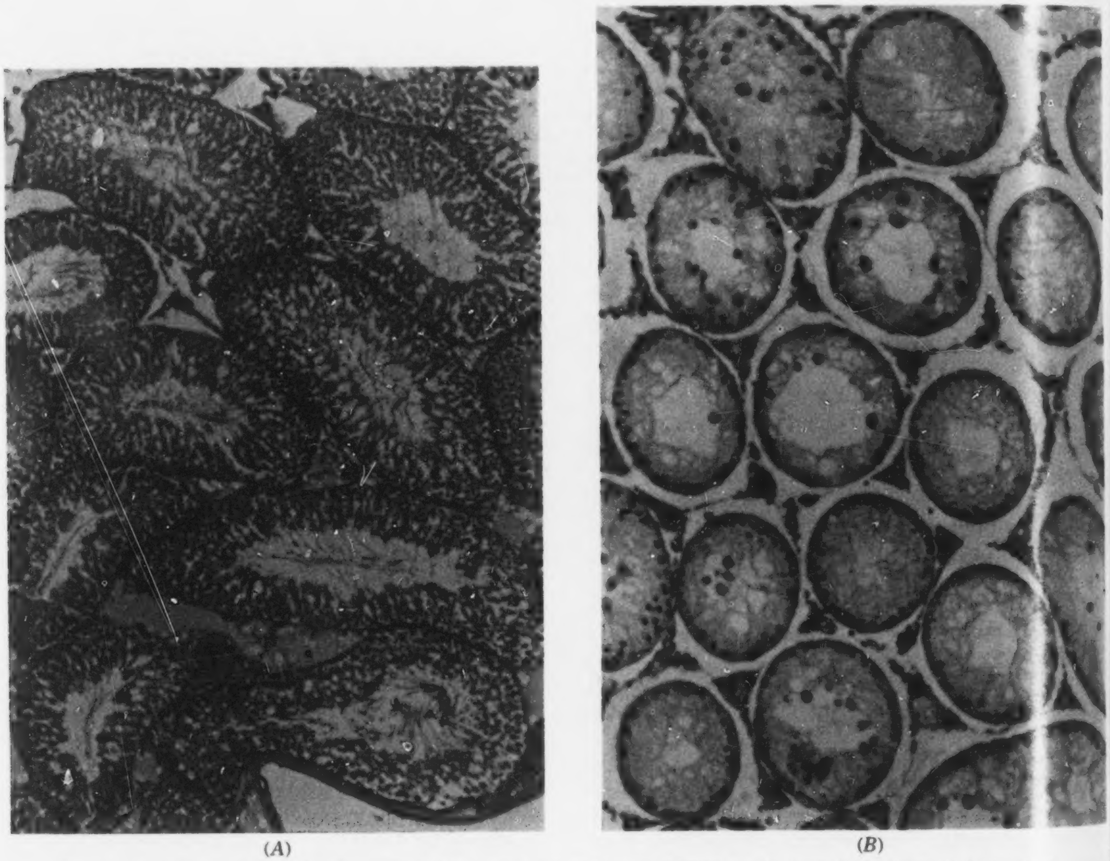

(*A*) (*B*)

FIGURE 1. Section of testicular tissue from (*A*) control rat; (*B*) rat on DEHP (2% in diet) for 90 days. H & E, 16 .

Investigations into the likely mechanisms involved showed that DBP-induced testicular injury did not result from the accumulation of metabolites or the formation of covalent adducts in testicular tissue. Metabolic disposition studies following oral administration of ^{14}C-labeled DBP showed no evidence of accumulation of radioactivity in the gonads. Furthermore, the testicular atrophy did not appear to be mediated by an interference in androgen synthesis or the availability of gonadotrophins. T eatment with testosterone or pregnant mare serum gonadotrophin did not reverse DBP-induced sticular injury (J. B. Gray, unpublished observat n).

Measurement of urinary zinc levels b atomic absorption spectrophotometry showed th t following DBP treatment there was an increa e in the urinary excretion of this element, kno n to be essential for testicular function (*8*). St dies us-

Table 2. Effect of DBP or MBP treatment on the relative testes weight in the rat.

Treatment	Dosage, mg/kg/day	Relative testes weight, % of control	
		4 days treatment	6 days treatment
Control ([illegible])		100	100
DBP	500	103	82^a
	1000	72^b	68^c
	2000	66^c	57^c
MBP	400	78^b	64^c
	500	66^c	53^c

[a] Significantly different from control, $p < 0.05$.

[b] Significantly different from control, $p < 0.01$.

[c] Significantly different from control, $p < 0.001$.

Table 3. Effect of DBP treatment on the urinary excretion and testicular content of ^{65}Zn in the rat.[a]

Treatment time, days (2000 mg/kg body weight)	^{65}Zn content, % of control	
	Urine (24 hr collection)	Testes (per 100 mg tissue)
2	125	88
4	175	77
6	125	64

[a] Animals were treated with $^{65}ZnCl_2$ 2 days prior to commencement of DBP treatment.

ing ^{65}Zn showed that DBP or MBP treatment led to a marked increase in the urinary excretion of radioactivity and a decrease in ^{65}Zn associated with testicular tissue (Table 3). Furthermore, the loss of testicular zinc in DBP-treated rats was accompanied by a decrease in the activities of two enzymes containing zinc, namely, alcohol dehydrogenase and carbonic anhydrase.

Studies on the testicular effects of monoalkyl phthalates derived from butyl alcohol isomers (*9*) showed that except for the tertiary butyl ester which was inactive, mono isobutyl and secondary butyl phthalates were as effective as MBP in producing testicular atrophy in the rat. Additionally, only those isomers producing testicular damage were found to alter zinc metabolism by increasing the urinary excretion of zinc and by depleting the concentration of this element in testicular tissues (Table 4). Investigations on the mono-*n*-butyl esters of phthalic acid isomers showed that whereas the *ortho* acid ester damaged testicular tissues in the rat, the *meta* and *para* derivatives were inactive.

Species sensitivity studies provided evidence that the rat, mouse, guinea pig and ferret were susceptible to testicular injury from DEHP and DBP (Table 5). However, the hamster appeared to be resistant to the gonadal effects of these compounds at the dose levels investigated. Furthermore, the corresponding monoesters were also found to be ineffective. A possible explanation for this finding in the hamster will be given later in the text.

As the formation of monoesters is an important metabolic step in the intestinal absorption and gonadal toxicity of oral ingested phthalates *in vitro* hydrolysis studies were conducted on a range of dialkyl phthalates employing small intestinal mucosal preparations from the rat, ferret, baboon and man (*10*). The results (Table 6) showed that the rate of hydrolysis of a dialkyl phthalate to the corresponding monoester was similar in the experimental

Table 4. Effects of butyl alcohol isomer monophthalates on the testes and zinc-65 metabolism in the rat.[a]

Treatment	Relative testes weight (g/100 body weight)	^{65}Zn content		Testicular injury[b]
		Testes, cpm/100mg tissue	Urine, cpm/24-hr specimen	
Control	0.975 ± 0.820	1951 ± 140	5869 ± 952	–
Mono-*n*-butyl phthalate	0.759 ± 0.127^e	1749 ± 138^e	6901 ± 221^b	+
Mono-*sec*-butyl phthalate	0.630 ± 0.074^e	1712 ± 147^e	7583 ± 209^e	+
Mono-isobutyl phthalate	0.708 ± 0.081^e	1809 ± 132^c	9620 ± 549^e	+
Mono-*tert*-butyl phthalate	1.026 ± 0.093	2000 ± 246	6078 ± 202	–

[a] Animals were pretreated with $^{65}ZnCl_2$ 48 hr prior to monophthalate treatment (800 mg/kg/day for 4 days).

[b] Testicular injury: – no change from control; + atrophy of seminiferous tubules.

[c] Significantly different from control, $p < 0.05$.

[d] Significantly different from control, $p < 0.01$.

[e] Significantly different from control, $p < 0.001$.

animal species examined, and furthermore, that with increasing length of the alkyl chain the hydrolysis rate decreased. Similar findings were obtained on two human biopsy specimens of small intestine.

Investigations on the testicular effects of a series of di-*n*-alkyl phthalates, ranging from C_1 to C_8, in the rat (*11*) showed that whereas the butyl, pentyl and hexyl compounds produced testicular injury, the lower and higher members of the series were inactive (Table 7). Furthermore, in agreement with the earlier finding that phthalate-induced testicular injury was accompanied by an adverse effect on gonadal zinc metabolism, only with those compounds found to act on the male rep oductive system in the rat was there as an sociated increase in the urinary excretion of zi c and a depletion of this element in the testes. T e reason why only three esters of the range of i-*n*-alkyl phthalates investigated produced testic ar atrophy is unclear and remains to be elucidat l. Intestinal hydrolysis rates does not appear be the determining factor as treatment with the m noesters of the inactive compounds were also ine ective in producing gonadal injury.

Di-*n*-pentyl phthalate (DPP) was found o be the most potent of the compounds investig ed, and testicular injury occurred within 24 hr f lowing a single oral dose in the rat. Histological udies on the early changes produced by DPP (*1*) showed that within 3 hr of treatment there was ev dence of incipient testicular injury characterized by the dissociation of germ cells from the basal membrane of seminiferous tubules. This effect was marked at 6 hr and progressed at 24 hr to the vacuolation of Sertoli cell cytoplasm (Fig. 2). E.M. studies confirmed that the main site of injury in the seminiferous tubules was the Sertoli cells and histochemical succinate dehydrogenase activity in the mitochondria was markedly reduced (Figs. 3 and 4). Further

Table 5. Testicular effects of phthalate esters in other species of experimental animals.[a]

Species	Compound	Findings[b]
Mouse	DBP; DEHP	+
Guinea pig	DBP; DEHP	+
Ferret	DEHP	+
Hamster	DBP; DEHP	–

[a] Dosage and duration of phthalate treatment: 2000 mg/kg/day, oral for 10 days.

[b] Findings: + relative testes weight reduced; histological evidence of testicular injury.

Table 6. *In vitro* hydrolysis of some di-*n*-alkyl phthalates by small intestinal mucosal cell preparations from the rat, ferret, baboon and man.

	Amount of product formed, μmole/hr/mg intestinal cell protein			
Phthalate diester	Rat (4) ($n=4$)	Ferret (3) ($n=3$)	Baboon (4) ($n=4$)	Man[a] ($n=2$)
Dimethyl	1.14 ± 0.07	0.05 ± 0.0	6.67 ± 1.16	111;2
Diethyl	0.648 ± 0.035	0.053 ± 0.017	4.33 ± 0.93	31.2;15
Di-*n*-butyl	0.590 ± 0.020	0.063 ± 0.008	2.19 ± 0.42	29.1;10
Di-*n*-octyl	0.219 ± 0.018	0.083 ± 0.026	0.190 ± 0.024	5.8;35 3

[a] Results expressed as nanomoles product formed per mg of total protein.

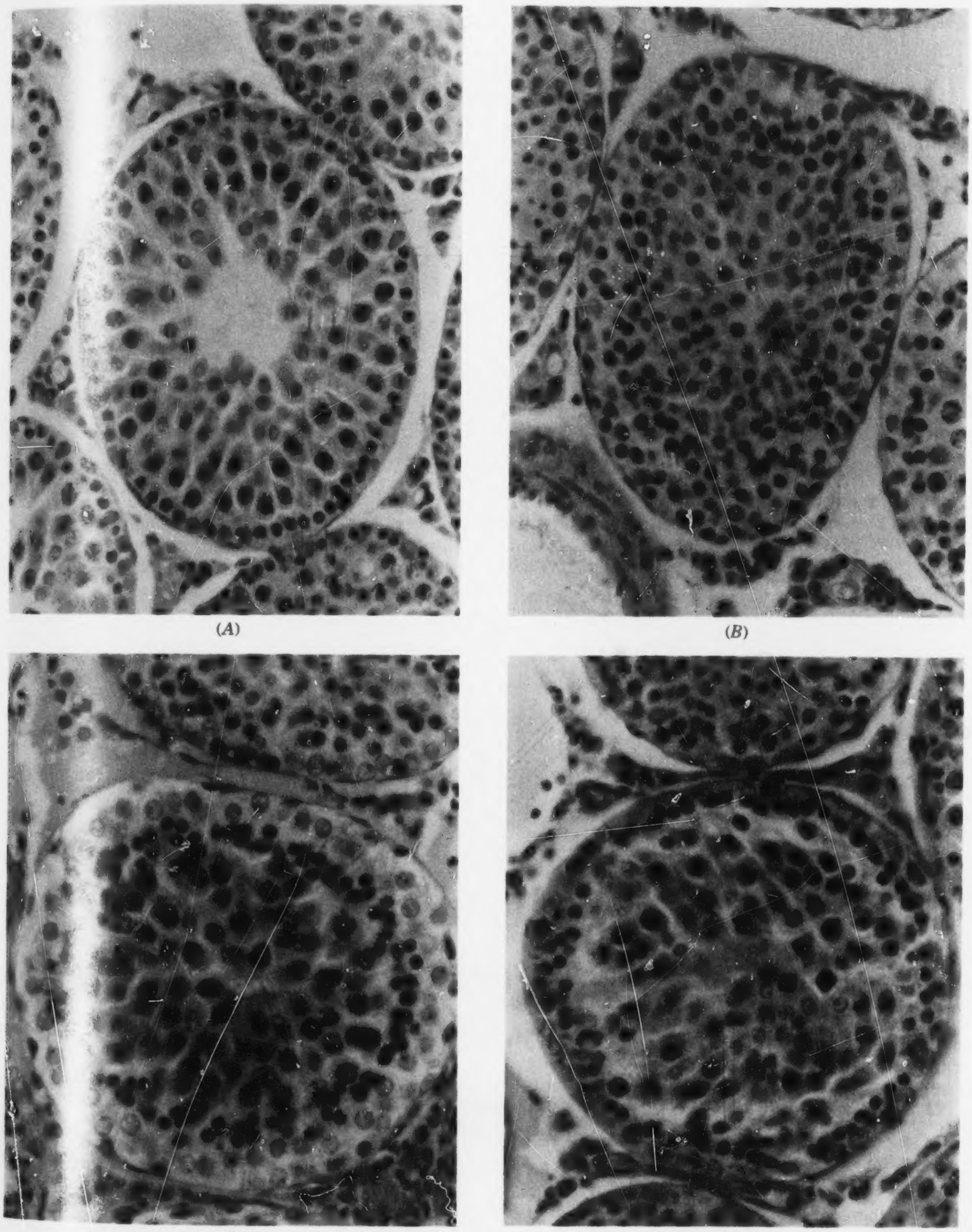
Table 7. Testicular effects of a number of di-*n*-alkyl phthalates in the rat.[a]

Phthalate diester				^{65}Zn content, % of control	
Type	Dose, g/kg body weight	Testicular injury[b]	Relative testes weight, % of control	Urine	Teste
Control (corn oil)	—	–	100 ± 2.1	100 ± 3.0	100 ± .5
Dimethyl	1.4	0	111 ± 2.9	93.2 ± 10.0	115.2 ± .8
Diethyl	1.6	0	98.6 ± 3.4	78.8 ± 4.1	89.1 ± .3
Di-*n*-propyl	1.8	0	106.7 ± 2.4	90.7 ± 2.3	95.5 ± .6
Di-*n*-butyl	2.0	+	66.9 ± 6.3[c]	138 ± 5.0[c]	53.1 ± .3[c]
Di-*n*-pentyl	2.2	+ +	56.6 ± 1.3[c]	139 ± 7.1[c]	38.0 ± .5[c]
Di-*n*-hexyl	2.4	+	64.8 ± 3.0[c]	133 ± 2.6[c]	49.7 ± .1[c]
Di-*n*-heptyl	2.6	0	102.7 ± 2.5	101 ± 1.1	98.6 ± .0
Di-*n*-octyl	2.8	0	106.3 ± 2.3	85.0 ± 5.7	104.5 ± .9

[a] The compounds were given orally at a dose of 7.2 mmole/kg/day for 4 days to young male animals.

[b] Testicular injury score: (0) no effect; (+) atrophy; (+ + +) severe atrophy.

[c] Significantly different from control, $p<0.001$.



(A) (B)

(C) (D)

FIGURE 2. Sections of testicular tissues from rats treated with a single oral dose (2000 mg/kg) of di-*n*-pentyl phthalate. (*A*) at time 0 (control); (*B*) at 3 hr; (*C*) at 6 hr; (*D*) at 24 hr. H & E, 400×.

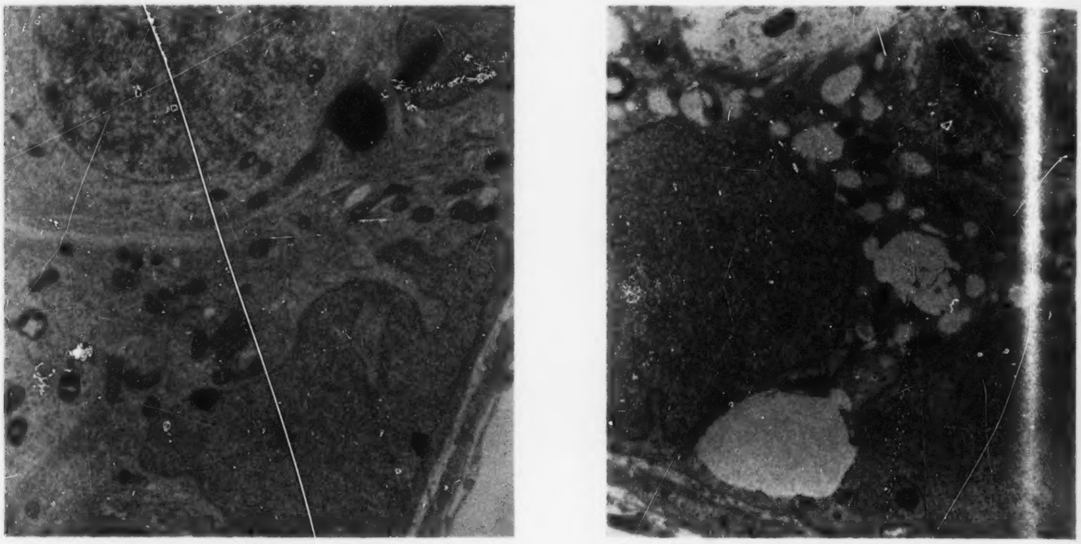

(*A*) (*B*)

FIGURE 3. Electron micrographs of seminiferous tubule from (*A*) control and (*B*) rat 6 hr after a single oral dose of di-*n*-pentyl phthalate (2000 mg/kg).

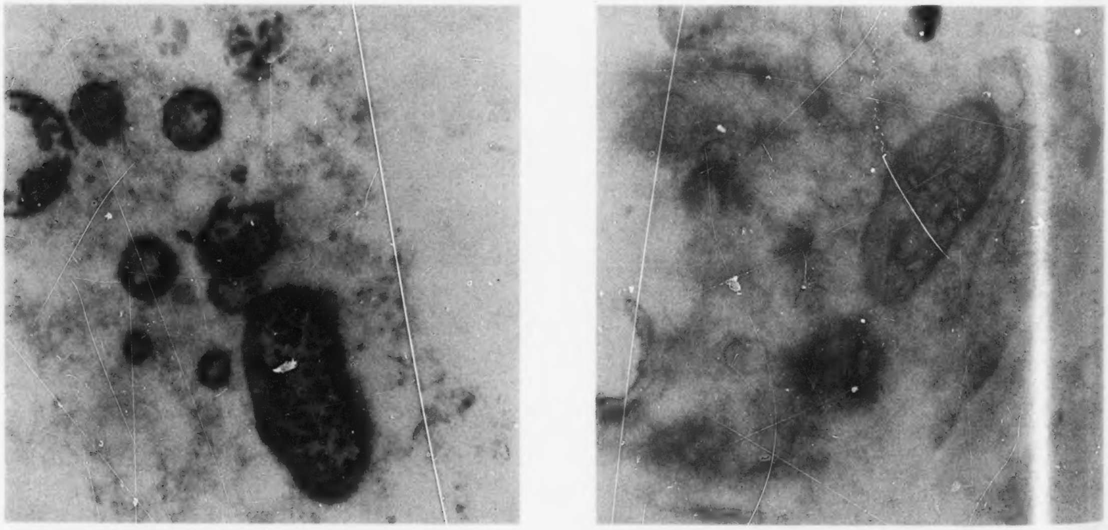

(*A*) (*B*)

FIGURE 4. Electron micrographs of histochemical succinate dehydrogenase activity in the Sertoli cells of seminiferous tubules from (*A*) control and (*B*) rat 6 hr after a single oral dose of di-*n*-pentyl phthalate (2000 mg/kg).

evidence that the Sertoli cells were the main target site was obtained from the finding that phthalate treatment reduced the production of rete testes fluid and of androgen-binding protein (T.B.G Gray, unpublished observation).

The rapidity in the onset of testicular atrophy and the characteristic changes produced in the seminiferous tubules suggested the possibility that tubular cell preparations in culture may respond to phthalates in a manner similar to the intact animal.

Table 8. Effect of DEHP, MEHP or 2-ethylhexanol on the dissociation of germinal cells from Sertoli cells in cultures of seminiferous tubule cell preparations.

Treatment	Total number of germinal cells released (× 10^6)	
	24 hr exposure	48 hr exposure
Control	5.8 ± 0.7	4.0 ± 0.5
DEHP (200 μM)	5.8 ± 0.2	3.9 ± 0.9
2-Ethylhexanol (200 μM)	5.3 ± 0.7	3.6 ± 0.6
MEHP (200 μM)	18.6 ± 2.0[a]	18.3 ± 3.0[a]

[a]Significantly different from control, $p < 0.001$.

Seminiferous tubules from rat testes were treated with trypsin and collagenase, and the preparations so obtained of Sertoli cells associated with germ cells were maintained in culture with Eagles minimum essential medium. In the first series of experiments, investigations were conducted on the behavior of these cells in culture on exposure to DEHP, mono(ethylhexyl) phthalate (MEHP) and 2-ethylhexanol. The results (Table 8) showed that whereas DEHP and 2-ethylhexanol did not increase above the background value the dissociation of germ cells from Sertoli cells, exposure to MEHP very markedly did so. Thus, MEHP appeared to act directly on seminiferous tubular preparations in culture.

Extending these studies to other monophthalates showed (Table 9) that the compounds causing testicular injury *in vivo* effected a concentration-related increase in the dissociation of germ cells from seminiferous tubule preparations in culture. Conversely, nonactive phthalates were ineffective at the concentrations examined. Interestingly, it has been found that preparations obtained from hamster testes do not respond on exposure to MEHP or MBP. These *in vitro* findings, in agreement with the observed resistance of the hamster to DEHP- or DBP-induced testicular injury suggest that the testicular tissues in the hamster, in contrast to the rat, may have protective mechanisms against phthalate diesters and monoesters.

The close parallel in the response of testicular cell preparations in culture to phthalates and their effects in the intact animal indicates that this *in vitro* system may be of value, not only in studies for the elucidation of mechanisms, but also for screening compounds for testicular effects in experimental animals and in man. Studies directed to these ends are currently in progress.

Table 9. Effect of monoalkyl phthalates on the dissociation of germinal cells from Sertoli cells in cultures of rat seminiferous tubule preparation on 48 hr contact.

Compound	Germinal cells detached, % of control				
	10μM	30μM	100	300	1000
Mono(2-ethylhexyl) phthalate	181[a]	206[b]	452[b]		
Mono-*n*-pentyl phthalate	[c]	179[a]	223[b]	257[b]	474[b]
Mono-*n*-butyl phthalate		147[a]	188[b]	191[b]	270[b]
Mono-*tert*-butyl phthalate					
Monomethyl phthalate			[c]		
Monoethyl phthalate			[c]		

[a]Significantly different from control, $p < 0.01$.
[b]Significantly different from control, $p < 0.001$.
[c]No change from control.

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Environmental Health Perspectives
Vol. 45, pp. 85-88, 1982

MEHP/DEHP: Gonadal Toxicity and Effects on Rodent Accessory Sex Organs

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The phthalate acid esters (PAEs), and, in particular, di(2-ethylhexyl) phthalate (DEHP) and its monoester, monoethylhexyl phthalate (MEHP), can adversely affect rodent testes but only at high doses. Rat gonadal zinc levels can be decreased by the injection of DEHP, but not MEHP. The rat prostate gland seems to be particularly sensitive to PAE-induced zinc depletion. PAE-induced changes in male reproductive organs were more evident in the rat than in the mouse. Some of the effects of MEHP can be demonstrated *in vitro* since it can alter the uptake of ^{65}Zn in rodent gonads and accessory sex organs.

Introduction

There has been recently renewed interest in the gonadal toxicity exerted by certain of the phthalate acid esters (PAEs). After some early observations by Shaffer et al. (*1*) reporting that diethylhexyl phthalate (DEHP) could adversely affect rodent testes, little attention was directed toward such actions for nearly 30 years. In 1977, Gray et al. (*2*) examined the short-term toxic effects of DEHP in rats and reported that testicular weights were significantly decreased at higher dietary levels of this PAE. More recently, and examining one of the principle metabolites of DEHP, namely, monoethylhexyl phthalate (MEHP), Oishi and Hiraga (*3-6*) have begun to shed some light upon certain aspects of gonadal toxicity. Dietary DEHP or MEHP can cause severe testicular damage in rats (*3, 4*). Comparable damage was recorded in mouse testes (*5, 6*). These investigators also reported that zinc levels in testes obtained from rats previously injected with MEHP were reduced (*4*). Other phthalates can alter zinc excretion in rats (*7*). Gonadal testosterone levels appear to remain unchanged by MEHP (*4*), but DEHP seems to lower the secretion of testosterone in the rat (*8*).

Many of the toxicologic effects upon either the male or female reproductive system have been recently reviewed (*9*). Some of the more major actions of MEHP and/or DEHP upon the male and female reproductive system have been summarized on Table 1. Other speakers at this conference will deal more specifically with the effects of phthalates on the female reproductive system and upon its feto- or embryotoxicity. Previous studies from this laboratory reported that MEHP was not teratogenic in rabbits (*15*).

The present experiments were undertaken to further investigate the actions of MEHP and DEHP upon male reproductive organs of the sexually mature rat and mouse. Gonadal and accessory sex organ zinc and nucleic acids were examined in rodents previously treated with either of these phthalates. Efforts were made to evaluate the effects of these two phthalates in relation to dose, route of administration and whether or not PAE-induced actions could be elucidated *in vitro*.

Methods

Male mice (Swiss-Webster) and rats (Sprague-Dawley) with average body weights of 37 g and 250 g, respectively, were used in these investigations.

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None of the doses of phthalates used in these experiments caused any impairment of body weight.

DEHP was obtained commercially (ICN Pharmaceuticals, Plainview, N. J.), while MEHP was generously supplied by Travenol Laboratories (Deerfield, Ill.). Appropriate vehicle-treated controls were run concurrently and consisted of 0.9% NaCl for MEHP and peanut oil for DEHP-treated animals. Routes of administration were either subcutaneous or intraperitoneal with animals being sacrificed 24 hr after the final injection. Various *in vivo* and *in vitro* doses or concentrations were employed depending upon the experimental design.

Organs were analyzed for endogenous zinc by atomic absorption spectrophotometry (Perkin-Elmer 305B) after initially digesting tissues in concentrated nitric acid overnight. Some reproductive system organs were analyzed spectrophotometrically for DNA (*16*) and also for RNA levels (*17*). The *in vitro* experiments utilized tissue slices (approximately 30 mg) bathed in an incubating medium of Krebs-Ringer bicarbonate buffer (pH 7.4) containing MEHP (5 or 10 *nM*) and ^{65}Zn (0.01 μCi/ml). Incubations were carried out at 37°C for periods of 1 or 2 hr in a Dubnoff metabolic shaker. Tissue levels of ^{65}Zn were subsequently analyzed on a gamma counter and were corrected for decay and background. Statistical analyses were carried out where appropriate.

Results

Table 2 depicts the action(s) of either MEHP or DEHP upon mouse endogenous zinc and nucleic acids. Neither dose nor phthalate affected testicular zinc or DNA/RNA ratios. There was some evidence of MEHP toxicity at the highest dose, namely, 100 mg/kg, as evidenced by a 50% mortality (Table 2).

Extending the duration of administration to an overall period of 20 days led to some significant changes in certain reproductive organs (Table 3).

Table 1. Effects of phthalate acid esters (PAEs) on the male reproductive system.

Species	PAE	Effect	Reference
Rat	DEHP	Testicular degeneration	Shaffer et al. (*1*)
Rat	DOP	Decreased testes weight	Harris, (*10*)
Rat	DEHP	Early fetal death and semisterility	Singh et al. (*11*)
Ferret	DEHP	Testicular degeneration	Lake et al. (*12*)
Rat	DEHP	Testis histological damage	Gray (*2*)
Rat	DEHP	↓ Testes weight ↑ Testosterone ↓ Zinc	Oishi and Hiraga (*8*)
Rat	DEHP	Testicular atrophy	Foster (*7*)
Mouse	DEHP	↓ Testosterone ↑ Testes weight ↓ Zinc	Oishi and Hiraga (*3*)
Rat	DEHP	↓ Testes weight ↑ Testosterone ↓ Zinc	Oishi and Hiraga (*4*)
Rat	DMP	↓ Testes weight ↑ Zinc excretion	Cater (*13*)
Rat	MEHP, MBP, MIBP	Testicular atrophy ↓ Zinc ↑ Testosterone	Oishi and Hiraga (*5*)
Mouse	MEHP, MBP, MIBP	↓ Testosterone ↑ Testicular weight	Oishi and Hiraga (*6*)
Rat	DEHP, DA79P	↓ Testicular weight	Mangham et al. (*14*)

Table 2. Action of MEHP or DEHP (50, 100 mg/kg daily × 5, IP) on mouse testes.

Ester	Concn.	n[a]	Zinc, % of control	DNA/RNA ratio
MEHP	Control	6	—	+3.2
	50 mg/kg	6	–1	+2.7
	100 mg/kg	3[b]	–22	+3.0
DEHP	Control	6	—	+2.4
	50 mg/kg	6	0	+2.3
	100 mg/kg	6	–1	–2.7

[a] Number of animals/group.

[b] Of the six mice injected, three died.

Table 3. Gonadal responses of mice and rats to MEHP and/or DEHP.[a]

Ester	n[b]	Species	Anterior prostate, % of control		Testes, % of control	
			Weight	Zinc	Weight	Zinc
MEHP, 50 mg/kg	6	Mice	+3	+1	−3	+1
DEHP, 100 mg/kg	6	Mice	+9	−11	+7	−3
MEHP, 50 mg/kg	6	Rats	−18	−37[c]	−6	+9
DEHP, 100 mg/kg	5	Rats	−3	−34[c]	−6	−31[c]

[a]Animals were injected (IP) every other day for a period of 20 days.
[b]Number of animals/group.
[c]Significantly different from control (p ≤ 0.05).

Table 4. Effect of previously injected MEHP (50 mg/kg, IP, every other day for 20 days) on the *in vitro* assimilation of ^{65}Zn by mouse reproductive organs.

	n[a]	Organ	^{65}Zn uptake interval, % of control	
			1 hr	2 hr
Control	6	Testes	—	—
MEHP	6		+11	−34
Control	6	Anterior prostate	—	—
MEHP	6		+75	+149[b]

[a]Number of slices.
[b]Significant at $p \leq 0.05$.

Table 5. Action of MEHP on the uptake of ^{65}Zn by slices of rat testes and ventral prostate *in vitro*.

MEHP level, *mM*	n[a]	Uptake, % of control[b]	
		Testes	Prostate
5	8	+22	+57[c]
10	10	+108[c]	+74[c]

[a]Number of slices.
[b]Uptake at 1 hr.
[c]Significant ($p \leq 0.05$).

Such changes, however, were only seen in the rat. Comparable doses of either MEHP or DEHP failed to modify testicular zinc or prostate zinc in the mouse. It is interesting to note that while zinc concentrations were significantly reduced (p 0.05), gravimetric responses remained unchanged in the rat gonads.

In an effort to examine further the lack of sensitivity of the mouse to PAE-induced changes in reproductive organs, a series of *in vitro* experiments were undertaken. Table 4 reveals that when mice were injected with MEHP and the organs subsequently examined for their ability to assimilate ^{65}Zn *in vitro*, that the prostates from the PAE-treated showed significantly higher levels of radioactivity. These prostate effects appeared to be time-related whereas no pattern of response was forthcoming in the mouse testes (Table 4).

The actions of MEHP *in vitro* were studied in the rat testes and prostate gland (Table 5). Using this *in vitro* experimental design rat zinc was again observed to be altered by MEHP. Both *in vitro* concentrations appeared to be effective in enhancing the assimilation of ^{65}Zn. The rat ventral prostate was somewhat more sensitive to MEHP-induced changes in ^{65}Zn assimilation than the testes (Table 5).

Discussion

The present studies, coupled with other recently published studies from this laboratory using rodent testes and prostate (*18, 19*), serve to confirm some of the previously reported effects of phthalates on the male reproductive system. The present studies, however, reveal several no-effect dose levels. More importantly, and based on the fact that the present studies employed nonoral routes of PAE administration, the action of either MEHP or DEHP in affecting depletion of endogenous gonadal zinc was not due to interference of the intestinal absorption of this divalent ion. Previous studies utilizing dietary MEHP or DEHP could not exclude this possibility as a mechanism for gonadal toxicity. A number of dietary constituents, including phytate, can interfere with the absorption of zinc (*20*).

The present studies tend to suggest that the mouse reproductive system is somewhat more resistant to the effects of the phthalates than the rat. The reason for this difference in gonadotoxicity is not evident, but it does not appear to be due to lower endogenous levels of testicular zinc in the rat.

The prostate gland, regardless of species, seems to be more profoundly affected by phthalates than does the gonad. Endogenous zinc can be depleted by both MEHP and DEHP in the rat ventral prostate. Only DEHP caused a slight reduction in endogenous zinc in the anterior lobe of the mouse prostate. However, the assimilation of ^{65}Zn by the mouse prostate and by the rat prostate was particularly enhanced by MEHP. Among the various zinc-containing organs in the mammal, the prostate reportedly contains relatively high levels of this ion (*21*). Zinc, along with a number of other divalent ions, can interfere with the binding of dihydrotestosterone to its receptor(s) in mouse prostate gland (*22*).

Exactly how the phthalates facilitate the turnover of endogenous zinc remains to be established. Phthalates can markedly increase the urinary excretion of zinc. It is possible that in the present studies that MEHP or DEHP facilitated the turnover of endogenous zinc thereby allowing increased incorporation of ^{65}Zn into the prostate or testes. Whatever the mechanism of phthalate-induced modifications in zinc metabolism, the present studies reveal that MEHP or DEHP can exert such actions both *in vivo* and *in vitro*.

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Environmental Health Perspectives
Vol. 45, pp. 89-97, 1982

Dimethoxyethyl Phthalate: Embryopathy, Teratogenicity, Fetal Metabolism and the Role of Zinc in the Rat[*]

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A single intraperitoneal injection (0.6 ml/kg) of dimethoxyethyl phthalate (DMEP) was given to groups of Wistar strain rats on day 10, 11, 12, 13 or 14 of gestation. Control rats received 0.6 ml/kg of physiological saline intraperitoneally.

In phthalate-treated rats, embryopathy was manifested by a high incidence (12-79%) of fetal deaths and fetal resorptions. Fetotoxic effects were expressed by a significant reduction in fetal weights. Hydrocephalus interna, a congenital malformation of the brain, was caused by DMEP. Congenital skeletal deformities (66-96%), with multiple skeletal (14-64%) and appendicular malformations (25-57%), were also induced by DMEP. Control rats exhibited no congenital malformations of the brain and no appendicular or multiple skeletal deformities.

DMEP caused a significant decrease in the zinc content of the fetus.

Fetoplacental metabolism 1 and 4 hr after intravenous administration of ^{14}C-DMEP suggested rapid transfer of the parent compound to the fetus across the placenta and that DMEP is a teratogenic moiety. The possible role of zinc in phthalate-induced teratogenesis in rats is also discussed.

Introduction

Plastics have become an integral part of our everyday lives. Phthalate esters are plasticizers widely used in the manufacture of plastics (*1*). In the United States, an estimated one billion pounds of phthalate esters are produced annually (*2*, *3*). There are approximately 20 different phthalate compounds sold as vinyl plasticizers, and one of the most toxic of these is dimethoxyethyl phthalate (DMEP) (*3*).

Phthalate esters are found in the composition of floor tiles, various types of home furnishings, waterproof clothing, industrial tubing, food wrapping materials (*4*, *5*), and a variety of medical and paramedical devices, including heart valves, vascular grafting material, intrauterine devices, catheters, dialyzing units, blood sets and disposable syringes. Residues of phthalates have been found in milk (*6*), human tissues and blood plasma (*7*), bovine tissues and the hearts of dogs, rabbits and rats (*8*). Phthalates, in general, are colorless, high-boiling liquids, soluble in organic solvents but immiscible in water, and they are degraded very slowly in the ambient environment. Diethylhexyl phthalate (DEHP), for example, closely resembles organochlorine pesticides (DDT, PCB) in rate of uptake, storage, and biomagnification in a variety of aquatic organisms (*9*).

The U.S. Department of Health and Human Services, particularly the Food and Drug Administration (*4*, *10*) and the National Institute of Environmental Health Sciences (*11-13*) has, therefore, a continuing interest and concern regarding the safety of phthalates. This concern is reflected in the sponsoring of conferences on phthalate esters in

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[*] This paper was not presented at the Symposium on Phthalate Esters.

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1972 (*3*, *4*, *9*, *13*) and in 1981. Several reviews (*14-23*) presented the toxicity of phthalate esters. Although DMEP has been shown to be one of the most toxic phthalates, little is known regarding the teratogenicity caused by a single maternal injection of DMEP during the organogenetic period in the rat.

Dietary supplementation of zinc has prevented teratogenic effects in rats induced by the maternal administration of a chelating agent, ethylenediaminetetraacetic acid (*24*). Dietary deprivation of zinc, on the other hand, could induce embryopathic and teratogenic effects in the rat, which was otherwise resistant to the teratogenicity of EM-12, a stable analog of thalidomide (*25*). Although phthalate esters bear similarities to thalidomide in inducing congenital malformations in the chick embryo (*26*), the role of zinc, if any, in phthalate-induced teratogenicity has not been reported.

Metabolism studies on phthalates using radiotracer techniques have been conducted in adult rats (*27-31*). There is only one report concerning maternalfetal transfer of radiolabeled phthalates. It deals with ^{14}C-DEHP and diethyl phthalate (*32*). Fetal metabolism of ^{14}C-DMEP, however, is unknown. The present studies, therefore, were designed to determine whether (a) maternal administration in rats of a single dose of DMEP during the organogenetic period induces embryopathic, fetotoxic or teratogenic effects; (b) multiple congenital skeletal malformations significantly higher than that of the control could be induced by DMEP; (c) uptake of ^{65}Zn by and the zinc content in the fetus are altered by administration of DMEP; and (d) DMEP reaches the fetus as a metabolite or as the unaltered parent compound.

Materials and Methods

Teratological Studies

Outbred female rats of the Wistar strain weighing 240-280 g were used. One female was placed with each male in a separate cage. The dropping of the mating plug from the female on the morning following mating was counted as day zero of the pregnancy, at which time the females were allocated to control and treatment groups. All animals had free access to a high protein pelleted rat diet (supplied by BP Nutrition, Stepfield, Witham, Essex, England) and water and were housed in a temperature-controlled (22 ± 2°C) animal facility.

DMEP of analytical grade was administered intraperitoneally (IP) at a dose of 0.6 ml/kg body weight on day 10, 11, 12, 13 or 14 of gestation. The control animals received the same volume (0.6 ml/kg) of physiological saline intraperitoneally. The number of animals in each treatment group is presented in Table 1.

Pregnant rats were anesthetized by chloroform on day 20, and the fetuses were removed by cesarian operation. More than half of the fetuses were assigned for evaluation of skeletal abnormalities (*33*, *34*); the rest were assigned for detection of organ malformations. The fetuses were prepared for evaluation of soft tissue abnormalities after fixation in Bouins fluid for one week (*35*) and then in 70% ethanol for 5 days. Wilson's technique was used for sectioning the fetuses and to evaluate gross organ pathology (*36*). The fetuses were prepared for evaluation of the skeletal abnormalities by the modified rapid clearing technique using the KOH-Alizarin Red S method of Staples and Schnell (*37*). Modifications included using 70% industrial methylated spirit (ethanol 94.5%; methanol 4.0%, water 1.5% *v/v*) in place of ethanol for fixation, leaving fetuses for 3-4 days in 1% KOH containing 0.01% Alizarin Red S stain for maceration and staining and keeping fetuses in clearing fluid for 2 to 3 days before transferring to an ethanol:glycerol (1:1, *v/v*) mixture for evaluation. The skeletons were evaluated and were classified as normal or retarded (*38*, *39*).

Table 1. Fetotoxicity of DMEP in rats: reduction in fetal weight.

Treatment[a]	No. of animals	No. of fetuses	Mean fetal weight (± SEM), g	d ($p = 0.01$), g[b]
Normal saline controls	17	157	4.09 ± 0.049	—
DMEP-10	19	49	2.87 ± 0.095[c]	0.15
DMEP-11	14	137	2.78 ± 0.027[c]	0.10
DMEP-12	12	113	3.04 ± 0.062[c]	0.14
DMEP-13	10	123	2.99 ± 0.029[c]	0.13
DMEP-14	15	102	3.34 ± 0.058[c]	0.21

[a] The number after DMEP refers to the day of gestation on which DMEP was injected.

[b] Minimum weight difference required for the mean fetal weight of the treated rats to be significantly different from that of the controls at $p = 0.01$ using Dunnetts test.

[c] Reduction in fetal weight was highly significant ($p < 0.01$).

Uptake of ^{65}Zn by and Zn Content in the Placenta and Fetus

DMEP or physiological saline was injected IP on day 13 of gestation at 0.6 ml/kg 4 hr prior to the administration of carrier-free $^{65}ZnCl_2$ in the coccygeal vein at a dose of 15 μCi/ml per kilogram of body weight. The animals were sacrificed by decapitation at 15 min, 1 hr and 4 hr after administration of the radiotracer zinc. The uptake of ^{65}Zn was measured with a Packard autogamma counter. The concentrations of zinc in the placental and fetal tissues were measured by atomic absorption spectrometry with a method (*40*) which was reliable and repeatable to 0.2 ppm of zinc. This experiment included 24 pregnant female rats, with four rats at each time period within a treatment group.

Fetoplacental Metabolism of ^{14}C-DMEP

^{14}C-DMEP, specific activity 0.912 mCi/mmole (2.025 × 10^7 DPM/mmole), was prepared from [carbonyl ^{14}C] phthalic acid (supplied by Radiochemical Centre, Amersham, Bucks, England). Radiochemical purity of the labeled ^{14}C-DMEP was ⩾ 99% as confirmed by gas-liquid and thin-layer chromatography. Chemical identity was confirmed by NMR and GC/MS.

Rats were injected intravenously on day 13 of gestation with ^{14}C-DMEP or physiological saline at 0.6 ml/kg body weight, and the animals were sacrificed by decapitation at 1 and 4 hr after treatment. Each treatment group had three animals. Placentae and fetuses were dissected out. For measurement of total phthalate concentration, pooled portions of placental (0.33 ± 0.10 g) and fetal tissues (0.50 ± 0.12 g) were dissolved in 1 ml Soluene and 10 ml of Instagel (both obtained from Packard Instrument Co.). For acid and neutral extractions of DMEP, portions of the placentae (0.40 ± 0.08 g) and the fetuses (0.[illegible] ± 0.18 g) were homogenized with 1 ml of 0.9% NaCl in a ground glass homogenizer. The homogenates were extracted three times with ethyl acetate ([illegible] ml). The extractions were separated into organic and aqueous phases by centrifugation (4000 g, [illegible] min). The residual aqueous phase was acidified to pH 1.0 with 2*N* H_2SO_4 and then reextracted with ethyl acetate. The neutral and acid extracts were made to 10 ml with ethyl acetate. A portion ([illegible] ml) of each extract was transferred to a scintillation vial and was evaporated under a stream of N_2 at [illegible]°C to a volume of ⩽ 1 ml before adding 10 ml of Instagel for counting. The chemiluminescence of the samples was minimized by storing the samples in a dark, cold storage room for 4-7 days. Counts, counting efficiency, and the original weight of the sample were used to calculate the radioactivity of each specimen in terms of disintegrations per minute per gram of tissue. These values were used in conjunction with specific activity of injected ^{14}C-DMEP to estimate molar concentrations of the teratogen in the fetus and the placenta.

Statistical Procedures

The reduction in mean fetal weight following administration of DMEP was compared with the normal saline controls by using Dunnett's procedure (*41*). Chi-square with Yates's continuity correction (*42*) was used to test the hypothesis that the percentages of dead and/or resorbed fetuses are equal in treatment and control groups and to compare the percentages of skeletally malformed fetuses with the control. Since the expected frequencies of the congenital deformities of the brain were small, Fisher's exact test was used to test these data. Student's *t*-test was used to compare the mean uptake of ^{65}Zn by and the Zn content of the placentae and the fetuses.

Results

Embryopathy, Fetotoxicity and Teratogenicity of DMEP

Maternal administration of DMEP in rats caused a significant ($p < 0.01$) reduction in the mean weight of living fetuses as compared to the corresponding value for the physiological saline-treated controls (Table 1). A single injection of DMEP induced a pronounced ($p < 0.01$) fetotoxicity during the organogenetic period regardless of the day of injection. The fetotoxic (Table 1) and the embryopathic (Table 2) effects were greater during the early stages (days 10 or 11 of gestation) of organogenesis than during the later stages (days 12, 13 or 14 of gestation).

Embryopathic effects were evaluated by percentage of dead and resorbed fetuses (Table 2). Reproductive toxicity of DMEP, in this regard, was four to seven times higher upon injection on day 10 of gestation as compared to days 11-14; 79% of the fetuses were dead and resorbed following maternal administration of DMEP on day 10. Intraperitoneal administration of normal saline to pregnant rats resulted in fetal death and resorptions in 7.6% of fetuses.

In comparison to controls, DMEP induced higher rates of congenital deformities of the brain. None of the fetuses from the control group exhibited hydro-

Table 2. Embryopathic effects of DMEP in rats.

Treatment	No. of rats	Total implantations	Number (%) fetuses, dead or resorbed	p values[a]
Normal saline controls	17	170	13 (7.6)	
DMEP-10	19	229	180 (78.6)	<0.[illegible]01
DMEP-11	14	175	38 (21.7)	0.[illegible]04
DMEP-12	12	141	28 (19.9)	0.[illegible]27
DMEP-13	10	139	16 (11.5)	0.[illegible]58
DMEP-14	15	121	19 (15.7)	0.[illegible]83

[a] The level of probability at which percent of dead or resorbed fetuses following administration of DMEP differed significantly from that of the control by the chi-square test.

cephalus interna, a malformation of the CNS, as opposed to a 13-26% rate of malformation in DMEP-treated animals (Table 3).

The incidence of congenital skeletal malformations caused by DMEP is presented in Table 4. Control rats exhibited an 18% incidence of skeletal malformations whereas DMEP-induced skeletal deformities were found in 66-96% of the fetuses. This rate of skeletal dysmorphogenesis was 3.5- to 5-fold higher than that in the controls. Fetuses in the control group showed no multiple malformations or appendicular dysmorphogenesis, while maternal administration of DMEP caused congenital multiple skeletal malformations in 14-64% of the fetuses and appendicular deformities in 25-57% of the fetuses. Malformations in the appendicular fibula were represented by its retardation, or by its absence. DMEP caused significantly higher ($p < 0.005$) rates of congenital skeletal deformities and multiple malformations in fetuses at all stages of gestation. Malformations in the fibula were observed upon administration of DMEP on day 12 or 13. The skeletal deformities induced by DMEP included complete loss of thoracic ribs, their lack of articulation from the spinal column and forked ribs with bending, cracking and cessation of the vertebral column at the lumbar or at the lower sacral region and the appendicular deformities as represented by the absence or marked shortening of the fibula.

Adverse Effect of DMEP on Maternal-Fetal Zinc Metabolism

The uptake of ^{65}Zn by placentae and fetuses and the zinc content in these tissues 1 hr post administration of ^{65}Zn are presented in Figures 1 and 2. There was no significant difference ($p < 0.05$) in ^{65}Zn uptake of the placentae and fetuses and the placental zinc content between DMEP and physiological saline treated animals. Fetal zinc content of rats treated with DMEP was, however, significantly lower ($p < 0.01$) than of those treated with physiological saline. The uptake and content of zinc in placentae and fetuses in other treatment groups (15 min and 4 hr) were not significantly different from the control.

Fetoplacental Metabolism of ^{14}C-DMEP

^{14}C-DMEP activity associated with the placenta and the fetus differed with respect to extraction (Table 5). Placental activity was associated mostly with the acidic metabolic products, since less than 10% (mean 6.4 ± 0.8) of the total activity was extractable from the neutral homogenates with ethyl acetate; while the remainder was recovered from the tissue residue after acidification. In contrast, 31-44.0% (mean 37.3 ± 4.5) of the fetal activity

Table 3. Congenital malformation of the brain produced by DMEP in rats.[a]

Treatment	No. of rats	No. of fetuses: Examined	No. of fetuses: Malformed	% fetuses malformed	p values[b]
Normal saline controls	17	26	0		
DMEP-10	19	14	2	14.3	0.117
DMEP-11	14	45	6	13.3	0.057
DMEP-12	12	55	13	23.6	0.004
DMEP-13	10	30	4	13.3	0.075
DMEP-14	15	39	10	25.6	0.004

[a] Hydrocephalus interna.

[b] Fisher's exact test was used for these data.

Table 4. Congenital skeletal malformations caused by DMEP in rats.

Variables	Saline controls	10	11	12	13	14
		Gestational age (days) at administration of dimethoxyethyl phthalate				
Fetuses examined	131	35	92	58	93	63
Fetuses malformed	23	31	61	50	89	57
Malformations, %	17.5	88.6	65.9	85.9	96.0	90.6
Significance[a]	–	<0.005	<0.005	<0.005	<0.005	<0.005
Fetuses multiple with malformations (no.)	0	21	13	37	31	27
Multiple malformations, %	0	60.0	14.1	63.7	33.3	42.8
Significance[a]	–	<0.005	<0.005	<0.005	<0.005	<0.005
Fetuses showing absence or retardation of fibula (no.)	0	0	0	33	23	0
% of fetuses showing malformations of fibula	0	0	0	56.9	24.7	0

[a] Significance denotes the level of p value at which percent malformations differs significantly from the control by Yate's continuity correction of chi-square test.

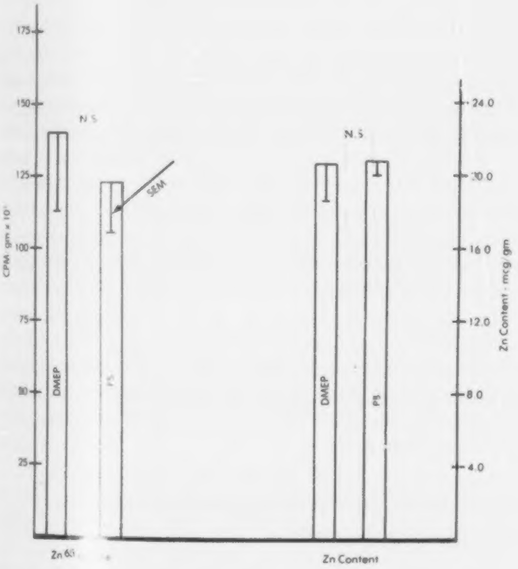

FIGURE 1. Mean uptake of ^{65}Zn by and the mean zinc content in 1 g of placenta 1 hr following administration of ^{65}Zn. Rats were pretreated with CMEP or physiological saline (PS) 4 hr prior to ^{65}Zn administration. Zinc content is in μg/g wet tissue. SEM is the standard error of the mean value based on four rats per treatment group. NS stands for not significantly ($p > 0.05$) different from PS group.

could be extracted, presumably as the unchanged diester, from the neutral homogenates with ethyl acetate 4 hr following administration of DMEP.

These preliminary results suggest, therefore, that the unmetabolized DMEP is transferred rapidly across the placenta to the fetus and the DMEP may be the teratogenic moiety.

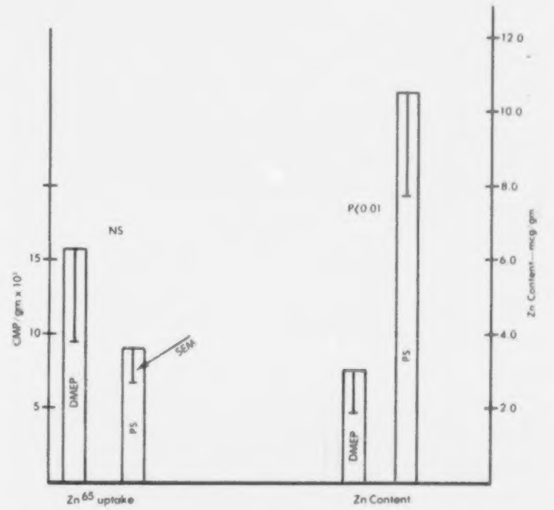

FIGURE 2. Mean uptake of ^{65}Zn by and the mean zinc content (μg/g wet tissue) in the fetus 1 hr following administration of ^{65}Zn.

Discussion

Teratogenicity of DMEP

The reported fetal deaths and resorptions caused by a single maternal administration of DMEP on day 10 of gestation in rats (Table 2) are in general agreement with the embryotoxicity resulting from multiple administrations of DMEP (*22*). A significant ($p < 0.01$) reduction in fetal weight (Table 1) has also been observed with other phthalate esters such as DEHP and dibutyl phthalate in rats (*43*) and in mice (*44*).

DMEP has been shown to be the most embryo- and fetotoxic member of the phthalate ester family (*3*,

Table 5. Metabolism of ^{14}C-DMEP in placental and fetal tissues of the rat.

Rat no.	Tissue	Time after administration, hr	DMEP concentration, nmole/g wet tissue		% DMEP
			Total	Neutral extract	
1	Placenta	1	2796.4	100.7	6
1	Fetus	1	1640	848.3	5 .7
2	Placenta	1	556.2	43.1	8
2	Fetus	1	368	101	2 .4
3	Placenta	4	396	30	6
3	Fetus	4	45.4	14	3 .8
4	Placenta	4	326.4	17.6	.4
4	Fetus	4	23.6	10.4	4 1
5	Placenta	4	376	29	.7
5	Fetus	4	40.9	13.3	3 .5
					$\underline{\overline{X}}$ = .4 ± 0.8 $\overline{X}$ = 3 .3 ± 4.5

16, 20). In part, this may be due to its high aqueous solubility compared to other phthalate esters, although the aqueous solubility of all phthalate esters is extremely low. DMEP, for example, is about 143 times (0.73 ml/100 ml H_2O) more soluble in water than DEHP (0.0051 ml/100 ml H_2O) and is the most soluble phthalate ester (*3, 21*). Fetal deaths and resorptions caused by DMEP were as high as 79% upon a single injection on day 10 of gestation (Table 2).

Congenital malformations of the brain involving hydrocephalus interna similar to those caused by DMEP (Table 3) have also been induced by dietary deficiency of zinc in rats (*45-51*). This is the first report to document hydrocephaly caused by the maternal administration of DMEP in rats.

Phthalate esters have been studied for their congenital skeletal dysmorphogenetic effects in rodents, but in this regard, these studies are the first to report skeletal malformations following a single injection of DMEP. Phthalate esters are considered compounds related to thalidomide (*26*). Congenital multiple skeletal malformations and appendicular dysmorphogenesis, which are considered "typical" of the thalidomide syndrome (*39*), have been difficult to produce in rats. These studies are the first, however, to report congenital multiple skeletal malformations and appendicular dysmorphogenesis following maternal administration of DMEP. Congenital multiple skeletal malformations upon a single intravenous maternal administration of thalidomide were also reported in our laboratory for the first time (*52*).

Intraperitoneal administration of sterile normal saline to pregnant animals resulted in some dead and resorbed fetuses and also caused skeletal malformations in control animals. Such effects have been reported earlier (*53*). The reproductive toxicity of DMEP, however, was several times higher than the normal saline control. The teratogenic effects of DMEP reported here have greater significance than the results where teratogenicity of phthalates is compared to untreated controls (*22*).

The malformations reported here are several hundredfold higher than the sporadic malformations found in rats, and are, therefore, due to the teratogenicity of DMEP. Major skeletal malformations in rats occur at a frequency of 0.41% (*54*), while those caused by DMEP were 161-234 times higher than this background level (Table 4). Craniofacial organ deformities such as hydrocephaly occur sporadically in rats at a frequency of 0.0506% (*54*), while DMEP induced this CNS malformation (Table 3) in 13-26% of the fetuses (257-514 times higher than the sporadic occurrence).

Role of Zinc in DMEP-Induced Teratogenesis

The conclusion that DMEP alters zinc metabolism in rats is based upon significantly (≤ 0.01) decreased zinc content in the fetuses of DMEP-treated rats as compared to that in the controls (Fig. 2).

Pregnant rats cannot mobilize zinc sufficiently from their body stores including that from the liver and bone (*50, 51*), to supply the needs of their fetuses. As a result of this insufficient zinc supply, a transient deficiency of zinc may have been produced following DMEP administration which, in turn, is reflected by the decreased zinc content in the fetal tissue. The teratogenic effects reported here are in agreement with those caused by the decreased zinc content in fetuses when rats were placed on zinc-deficient diets during gestation (*51*).

Permeability of the placenta to zinc increases after day 18 of gestation (*55*). However, this has not negated the validity of the results reported here because fetal zinc concentrations relate to maternal administration of DMEP or of physiological saline on day 13 of gestation. The fetal zinc concentration (micrograms per gram of wet tissue) reported here is far lower than that reported by Hurley and Swenerton (*51*) in 19- and 21-day-old fetuses. This may be due to the increased permeability of the placenta to zinc after day 18 of gestation and due to maternal supplementation of dietary zinc at 100 ppm, as well as other factors (*54*).

The results of the fetoplacental metabolic studies of zinc (Figs. 1 and 2) are also in agreement with the pharmacokinetic aspects of placental transfer of drugs using a single compartment model for dam and fetus, and in agreement with the first-order kinetics described by Levy and Hayton (*56*).

The adverse effects of DMEP on zinc metabolism and associated fetal anomalies reported here (Tables 1-4, Figs. 1, 2) permit one to draw an analogy between the phthalate-induced testicular damage in rats and the associated adverse effects on zinc metabolism caused by phthalates (*57, 58*). The authors postulate that the protection provided by zinc in phthalate-induced testicular damage (*57, 58*) may be analogous to the protection offered by zinc against certain teratogens in rats (*24*). Singh et al. (*32*) reported that first order kinetics describe the elimination of ^{14}C-labeled phthalates from maternal blood, amniotic fluid and fetal tissue with time. Also, zinc (*59*) and phthalates (*32*) follow, in general, a similar time course and concentration kinetics in maternal blood plasma and fetal tissue. Therefore, the authors propose a role of zinc in DMEP-induced teratogenesis in rats.

The reduced content of zinc in the target organ, the fetus (Fig. 2), following administration of DMEP, may be due to the rapid turnover rate of zinc following administration of phthalates (*57, 58*). Coadministration of zinc was found to offer a substantial measure of protection against testicular atrophy and loss of organ weight caused by dibutyl phthalate (*57*).

The protective effect, if any, of coadministration of zinc and the underlying mechanism of zinc against the fetotoxic and teratogenic effects of DMEP is unknown, as is the protective mechanism of zinc therapy in phthalate-induced testicular atrophy. There are many common parallels, however, between congenital abnormalities caused by zinc deficiency in rats (*45, 47-50, 60, 61*) and those produced by DMEP (Tables 1-4). These include the manifestation of fetotoxicity by a marked reduction in fetal weight (Table 1); embryopathy by fetal resorption (Table 2); congenital malformations of the brain (*45, 47, 48, 50, 60, 61*), namely, hydrocephalus interna (Table 3); and skeletal malformations (Table 4). The incidence of hydrocephalus interna produced by DMEP (Table 3) was similar to that produced by maternal zinc deficiency (*51, 60, 61*), although, in general, its severity was much less.

Fetoplacental Metabolism of ^{14}C-DMEP

Although no specific study has been reported which identifies the nature of DMEP or its metabolites in the rat fetus or placenta, the results reported here are in general agreement (*28-32, 62*) with the metabolism of phthalates in nonpregnant rats as obtained by using radiotracer techniques.

There is a relationship between teratogenicity and carcinogenicity (*63*). Such a relationship for DMEP, is not unequivocally established. Although further studies are required to confirm whether or not DMEP is a carcinogen, there are examples of a carcinogen having teratogenic potential and a teratogen having carcinogenic potential (*63*). This is so because congenital malformations occur through genetic defects, or through embryotoxic effects on prenatal development, or both. For example, methotrexate has carcinogenic properties and possesses teratogenic potential, while 6-mercaptopurine, cyclophosphamide, and nitrosamides are teratogenic and have carcinogenic potential (*63*). Several researchers (*3, 10, 12, 16, 26, 64, 65*) have therefore cautioned about the toxicity and possible health threats of phthalates.

Conclusions

The studies described here establish that the maternal administration of dimethoxyethyl phthalate (DMEP) in a single dose during the organogenetic period (days 10-14) in the rat causes: (a) embryopathic, fetotoxic and teratogenic effects several times higher than reported by the widely studied plasticizer, diethylhexyl phthalate (DEHP); (b) congenital deformities of the brain, such as hydrocephalus interna; (c) congenital skeletal malformations (66-96%), including multiple skeletal (14-64%) and appendicular deformities (25-57%); (d) adverse effects on maternal-fetal metabolism of zinc; and (e) teratogenicity, as mentioned above, due to DMEP in the fetus.

The authors express their appreciation to Dr. Thomas Connors, Director, MRC, Toxicology Unit; Dr. Gerald B. Guest and Dr. Terence Harvey, Director and Deputy Director, respectively, of the BVM FDA, for their continued interest and support during the course of investigations and thereafter; Dr. D. J. Campbell of MRC for the synthesis of ^{14}C-labeled DMEP, Dr. James Colaianne and Dr. Robert J. Condon, BVM, for their advice on statistical procedures; Dr. Maurice Zeeman for constructive suggestions, to Miss Kathy Briscoe for secretarial help; and to Mr. Malcolm King of the MRC Toxicology Unit for his excellent help and cooperation in the breeding, management, and handling of the laboratory animals used in this study.

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Environmental Health Perspectives
Vol. 45, pp. 99-101, 1982

Pht alate Ester Testing in the National Tox cology Program's Environmental Mut genesis Test Development Program

by E rol Zeiger,* Stephen Haworth,† William Speck‡ and K ristien Mortelmans**

A number of phthalate esters and related chemicals were tested for mutagenicity in *Salmonella typhimurium*. The chemicals were tested blind in three laboratories by a preincubation modification of the Ames Salmonella/mammalian microsome test using S-9 prepared from Aroclor-induced rats and Syrian hamsters. All chemicals tested were judged to be nonmutagenic.

Introduction

The Cellular and Genetic Toxicology Branch of the National Toxicology Program (NTP) is responsible for developing and validating new and established mutagenicity test protocols and methodologies and for testing chemicals of interest to the NTP for mutagenicity (*1*). All chemicals entering the progr m are tested in *Salmonella typhimurium* for the in luction of gene mutations, and selected chemical re tested further in *Drosophila melanogaster* f the induction of sex-linked recessive lethal m ations and reciprocal translocations, in cultured hinese hamster ovary cells for the induction of c omosome aberrations and sister chromatid exch ges, or other test systems.

A seri of phthalate esters and related chemicals were se ted for testing in Salmonella and other systems his report lists chemicals selected for testing d presents the Salmonella test results from th chemicals for which testing has been complet All tests reported here were done by three different laboratories under contract to the NTP: EG&G Mason Research Institute, Rockville, Maryland, under the direction of Dr. Stephen Haworth; Case Western Reserve University, Cleveland, Ohio, under the direction of Dr. William Speck; and SRI International, Menlo Park, California, under the direction of Dr. Kristien Mortelmans.

Methods

Protocol

All testing was done using a preincubation modification of the method of Ames et al. (*2*). Briefly, *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 (obtained from B. N. Ames, Berkeley, Calif.) were used with 9000 *g* liver homogenate preparations (S-9) from Aroclor 1254-induced male Sprague-Dawley rats and Syrian hamsters prepared according to the method of Ames et al. (*2*). A 100 μl portion of an overnight culture of cells was mixed with either 0.5 ml of S-9 mix or 0.1*M* pH 7.4 phosphate buffer, and 50 or 100 μl of test chemical or solvent in a tube. This mixture was incubated at 37°C for 20 min; 2.0 ml of top agar was added and the mixture poured onto a minimal agar plate and incubated at 37°C for 2 days, after which the histidine revertant colonies were machine counted.

Each chemical was tested at five doses separated

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Table 1. Phthalates and related chemicals tested in Salmonella.

Name	CAS #	Supplier (purity/grade)	Laboratory[a]	Solvent	Dose range (µg/plate)
Butyl benzyl phthalate	85-68-7	Monsanto (97%)[b]	EGG	DMSO	100-10000
			CWR	″	33-11550
Butyl cyclohexyl phthalate	84-64-0	—[c]			
Diallyl phthalate	131-17-9	Hardwicke (99%)	CWR	DMSO	100-10000
			EGG	DMSO	100-10000
Dibutyl phthalate	84-74-2	Aldrich (99%)	EGG	DMSO	100-10000
Dicyclohexyl phthalate	84-11-7	—			
Di(2-ethylhexyl) adipate	103-23-1	Pfaltz & Bauer (N.S.)[d]	EGG	95% EtOH	100-10000
			SRI	DMSO	100-10000
Di(2-ethylhexyl) phthalate	117-81-7	MC/B (Practical)	EGG	DMSO	100-10000
			CWR	DMSO	100-10000
		WR Grace (99.5%)[b]	SRI	DMSO	100-10000
			EGG	95% EtOH	100-10000
Diethyl phthalate	84-66-2	Chem. Tech. Ind. (99.9%)[b]	EGG	DMSO	10-3333
			CWR	DMSO	100-10000
Di(*n*-hexyl) phthalate	84-75-3	—			
Diisobutyl phthalate	84-69-5	Eastman (Reagent)	EGG	DMSO	100-10000
Diisodecyl phthalate	26761-40-0	Pfaltz & Bauer (NS)	SRI	95% EtOH	100-10000
			EGG	95% EtOH	100-10000
Diisononyl phthalate	28553-12-0	—			
Dimethyl phthalate	131-11-3	Aldrich (99%)	EGG	DMSO	33-6666
Dimethyl terephthalate	120-61-6	Aldrich (99%)	EGG	DMSO	3.3-333
Dioctyl phthalate	117-84-0	Eastman (98%)	SRI	DMSO	100-10000
Ditridecyl phthalate	119-06-2	Polysciences (NS.)	EGG	Acetone	100-10000
Diundecyl phthalate	3648-20-2	—			
2-Ethoxyethanol	110-80-5	Dow Chem. (99%)[b]	EGG	Water	100-10000
2-Ethyl-l-hexanol	104-76-7	Aldrich (99%)	EGG	DMSO	3.3-333
Mono(2-ethylhexyl) adipate	4337-65-9	—			
Mono(2-ethylhexyl) phthalate	4376-20-9	—			
Phthalamide	88-96-0	Sherwin Williams (99+%)[b]	EGG	DMSO	33-1500
Phthalic anhydride	85-44-9	Aldrich (99+%)	EGG	DMSO	1-666
Terephthalic acid	100-21-0	Eastman (98%)	SRI	DMSO	100-10000
Tetrachlorophthalic anhydride	117-08-8	MC/B (Prac.)	CWR	DMSO	33-6666.7
Tris(2-ethylhexyl)phosphate	78-42-2	Chem. Systems Lab (97.6%)[b]	CWR	DMSO	100-10000
Tris(2-ethylhexyl)phosphite	301-13-3	Pfaltz & Bauer (NS)	CWR	DMSO	100-8270

[a] Laboratory: EGG: EG&G Mason Research Institute; CWR: Case Western Reserve University; SRI: SRI International.

[b] Sample used for carcinogenesis bioassay.

[c] Chemical not yet received.

[d] NS = purity/grade not specified.

clearing of the background/lawn), the preferred high dose was 10 mg/tube. All tests were repeated at least one week following completion of the test. The positive controls, dissolved in DMSO, were without S-9: for TA98, 4-nitro-*o*-phenylenediamine; for TA100 and TA1535, sodium azide; for TA1537, 9-aminoacridine; with S-9 for all strains, 2-aminoanthracene. The solvents used were water, dimethyl sulfoxide or 95% ethanol. Concurrent positive and solvent controls were run with all experiments.

Chemicals

The chemicals selected for testing, supplier and purity or grade are listed in Table 1. Butyl benzyl phthalate, di(2-ethylhexyl) phthalate, diethyl phthalate, 2-ethoxyethanol, phthalamide, and tris(2-ethylhexyl) phosphate were obtained from the NCI Bioassay Repository; the other chemicals were purchased commercially.

Coding

All chemicals were tested as coded aliquots and were sent to the testing laboratories interspersed with unrelated chemicals, including known mutagens and nonmutagens, so that the testing laboratories were unaware of the identity of the chemicals or chemical classes under test. All testing and evaluation of data for any aliquot was completed and a decision made before the chemical was decoded. A positive mutagenic response was defined as a reproducible, dose-related increase in *his*$^+$ revertants over the spontaneous level.

Results

Testing has been completed in Salmonella for 20 chemicals, six of which were tested in more than one laboratory (Table 1). No mutagenic responses were seen with any of the phthalates tested in any of the laboratories. The concurrent positive controls produced the expected mutagenic results.

The di(2-ethylhexyl) phthalate tested came from two sources; the first was purchased commercially for this program; the second was received from the NCI Repository and was from the same batch as was used for the NCI Bioassay Program. The data from these tests will be published elsewhere after all testing is completed.

Discussion

A series of 20 phthalates and related chemicals were demonstrated to be nonmutagenic in a preincubation modification of the Salmonella microsome test. Butyl benzyl phthalate, di(2-ethylhexyl) adipate and di(2-ethylhexyl) phthalate were classified as carcinogens in the NCI Bioassay Program; phthalic anhydride was classified as noncarcinogenic.

There is an apparent disagreement between the results reported here and elsewhere in this issue. Two other studies, using different Salmonella protocols, have reported diethyl phthalate to be mutagenic, in disagreement with the results reported here: however, these other reports agree that di(2-ethylhexyl) phthalate is not mutagenic in Salmonella.

Based on the results from these three carcinogenic chemicals, it would appear that the Salmonella microsome test is not responsive to phthalates and would be a poor predictive test for their potential carcinogenicity. However, mutagenicity and carcinogenicity results from a larger number of phthalates are necessary before definitive statements can be made concerning the predictability of the Salmonella microsome test for carcinogenesis in rodents.

A number of these phthalates are currently on test or have been selected for testing in Drosophila for their ability to induce sex-linked recessive lethal mutations and heritable translocations and in cultured Chinese hamster ovary cells for induction of chromosome aberrations and sister chromatid exchanges.

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Environmental Health Perspectives
Vol. 45, pp. 103-109, 1982

Assessment of the Mutagenicity of Phthalate Esters

by Walter J. Kozumbo,* Rosanna Kroll* and Robert J. Rubin*

The Ames assay was used to investigate the mutagenicity of several phthalate esters as an approximation of their carcinogenic potential. The *ortho* diesters, dimethyl phthalate (DMP) and diethyl phthalate (DEP) produced a positive dose-related mutagenic response with Salmonella TA100, but only in the absence of S-9 liver enzymes. Dibutyl, di(2-ethylhexyl), mono(2-ethylhexyl), and butyl benzyl phthalate as well as the dimethyl isophthalate and terephthalates and the trimethyl ester, trimellitate, were not mutagenic with TA100 or TA98 in the presence or absence of S-9. In a host-mediated assay, extracts of 24-hr urines of rats injected IP with DMP (2 g/kg) were not mutagenic to TA100 at levels up to 8 equivalent-ml of urine/plate (representing 30% of their daily urinary output). *In vitro* studies revealed that S-9 associated esterase hydrolyzed DMP to the monoester and methanol and eliminated its mutagenicity. Whole rat skin was shown to have about 1.5% of the DMP-esterase activity of liver, when compared on a wet weight basis. An *in vitro* binding study indicated that epidermal macromolecules bound DMP at a severalfold greater rate than hepatic macromolecules. Thus, both the mutagenicity and binding of DMP are inversely related to the metabolism of this compound. These results suggest that skin could be at high risk for a mutagenic/carcinogenic insult.

Introduction

Since there are scant data on the mutagenic and carcinogenic potential of a variety of phthalate esters, the Ames assay was used to investigate the mutagenicity of some of these compounds as an approximation of their carcinogenic potential. This paper presents evidence for the mutagenesis of dimethyl phthalate (DMP) and diethyl phthalate (DEP), along with additional studies on the metabolism of DMP and its binding to macromolecules.

DMP is a widely used insect repellent which is available to the general public as an active ingredient in commercial products. In its pure form it is used by agricultural workers in doses up to 100 mg/kg applied directly to the skin (*1*) and by the U.S. military in tropical regions of the world. Considering the many people exposed to DMP as well as the potentially large doses to which they may be exposed, further research on the mutagenic effects of this chemical is well justified.

A literature search in the area of mutagenicity of DMP revealed relatively few studies. One study by Pilinskaya et. al. (*2*) reported no increase in chromosomal aberrations in bone marrow cells of white mice administered DMP intraperitoneally (dose not cited). Another study by Tsuchiya and Hattori (*3*) also reported no increase in chromosomal damage in human leukocytes treated in culture with 20 μg/ml of DMP. On the other hand, Yurchenko (*4*) did report a significant increase in chromosomal aberrations in hepatocytes from rats treated topically with 1250 mg/kg DMP five times per week for one month. Rats administered only a single topical dose displayed no significant increase in chromosomal aberrations. Thus, DMP's topical use as an insect repellent and the observation of hepatic chromosomal damage would suggest skin and liver, respectively, as potential sites for mutagenic/carcinogenic effects.

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Methods

Mutagenesis

The standard Ames bacterial mutagenicity assay (5) was used with several modifications as described by Batzinger et al. (6). The major modifications included shifting the histidine and biotin (at concentrations of 50 and 15 g/plate, respectively) from the top to the bottom agar, and reducing the glucose to 67.5 mg/plate in the bottom agar. Each phthalate ester was tested with *Salmonella typhimurium* strains TA98 and TA100 in the presence and absence of S-9 liver microsomal fraction from male Sprague-Dawley rats induced with Aroclor 1254.

Host-Mediated Assay

Male Sprague-Dawley rats (approximately 350 g) were injected IP with 2 g/kg of DMP and 24-hr urine was collected. The urine from 12 animals was pooled. Five such pools were collected. The urine (not further acidified) was extracted once with *n*-butanol which was then evaporated. The residue was dissolved in DMSO and tested in the Ames assay. Aliquots representing various equivalent volumes of urine from each pool were tested.

Chemical Analysis of Urine

The 24-hr pooled urine from a group of 12 rats (approximately 350 g) was acidified to pH 1-2 with HCl and then exhaustively extracted with *n*-butanol until no UV-absorbing phthalate moieties could be detected in the urine by direct thin-layer chromatographic (TLC) analysis. The butanol extracts were combined and examined by TLC. The UV absorbant spots corresponding to the R_f values of monomethyl phthalate (MMP) and DMP standards were scraped from the plates and extracted with methanol. The spectrophotometric absorbance of the methanol extracts at 275 nm was used to determine the amount of phthalate present. No other UV-absorbing spots were seen on the TLC plates.

Preparation, Incubation and Treatment of Skin Slices

The backs of male Sprague-Dawley rats between 300 and 400 g were shaved 24 hr prior to sacrifice by cervical dislocation. Dorsal skin was removed immediately after sacrifice and the fat and loose connective tissue adhering to the hypodermis was scraped away with a scalpel. The skin, now consisting of dermis and epidermis, was sliced, weighed and placed into phosphate buffered saline (PBS), pH 7.4, at a ratio of 1 to 5 (w/v). DMP was added to a final concentration of 1.5 mg/ml. The incubation mixture was shaken at 37°C for various time periods up to 2 hr before the reaction was terminated with the addition of sufficient 1.0*N* HCl to reduce the pH to approximately 1.5. The acidified medium was decanted and the skin slices were washed several times with PBS while maintaining the pH at 1-2. The washes were pooled with the original medium and the total volume was extracted with chloroform until no phthalate moieties could be detected by direct TLC analysis of the aqueous pooled fraction. In addition, the skin slices were also rinsed several times with chloroform. The chloroform extracts of both the skin slices and aqueous medium were pooled and centrifuged at 1000*g* for 10 min. The supernatant was then fractionated by TLC and the content of MMP and DMP determined as above.

Preparation, Incubation and Treatment of Liver S-9

A 0.15 ml aliquot of DMP (4000 μg/ml water) was added to 2 ml of E medium plus 0.5 ml of S-9 mix (5). The mixture was shaken at 37°C for various time periods up to 1 hr. The reaction was terminated by the addition of 0.4 ml of 1.0 *N* HCl. After centrifugation at 1000 *g* for 10 min, 2 ml of the acidified supernatant (pH 1.5) was extracted twice with chloroform.

Chemical Analysis of *In Vitro* Skin and Liver Extracts

The rate of *in vitro* metabolism of DMP to MMP and methanol was measured by the formation of MMP. The chloroform extracts from the liver S-9 and the skin incubations were analyzed for MMP by the TLC-spectrophotometric method described in the above section on chemical analysis of urine. The rate of metabolism was determined over the linear range of metabolism for each tissue.

In addition, the *in vitro* metabolism of DMP to methanol by skin slices was investigated using the basic method of Wood and Siddiqui (7). A slight modification of the method allowed for simultaneous determination of formaldehyde formation. This was accomplished by measuring the amount of formaldehyde formed in the presence and absence of $KMnO_4$, which is used to convert methanol to formaldehyde. In the presence of $KMnO_4$, the total of methanol plus formaldehyde is measured. In the absence of $KMnO_4$, only the presence of formaldehyde is detected. The difference between the two procedures yields the amount of methanol formed.

Preparation of Epidermal and Hepatic Homogenates

The homogenized epidermis was prepared by modification of previously described techniques (*8*). After the removal of the entire dorsal skin as described above, it was immersed in distilled water at 50°C for [illegible] sec and then immediately transferred to iced water for another half minute. The epidermal layer was then scraped off with a scalpel, suspended in 2-3 ml of ice cold PBS (pH 7.4) and homogenized. The homogenate was then strained through a fine nylon mesh.

A rat was decapitated and following exsanguination the *in situ* liver was perfused with 30-40 ml of PBS (pH 7.4) via the hepatoportal vein. The liver was then removed and homogenized in PBS at a ratio of 1 to 3 (w/v). The homogenate was strained through fine nylon mesh.

A 1-ml aliquot of each of epidermal and liver homogenates was mixed with 1 ml of ^{14}C-carbonyl-labeled DMP solution (3.0 mg/ml of DMP in PBS), representing from 1.4×10^5 to 2.0×10^6 dpm per vial. The incubation mixtures were shaken at 37°C for 2 hr and the reaction terminated and the macromolecules precipitated with the addition of 4.0 ml of 0.9*M* trichloroacetic acid (TCA).

Determination of Macromolecular Binding

To determine macromolecular binding, the method of Jollow et al. (*9*) was employed. Successive washings with TCA, 80% ethanol and acetone were continued until the 1 ml aliquots of the wash solution (dissolved in ACS Aquasol scintiliation fluid) produced counts equivalent to background on a Packard Tricarb liquid scintillation spectrometer.

The final acetone washed TCA precipitate was taken to dryness and weighed to determine the mg of dry weight. Finally, the macromolecular residue was solubilized in NCS tissue solubilizer (Amersham) for counting.

Results

The results from the modified Ames mutagenic assay of phthalic acid (PA), DMP and DEP in TA100 in the absence of S-9 are shown in Table 1. Hycanthone was used as the positive control; it consistently produced a response of greater than 600 net revertants at a concentration of 30 μg/plate. PA, the de-esterified product of phthalate esters, proved negative in this assay. However, both DMP and DEP registered a positive and dose-related response. At 1000 μg the mutagenic response rep-

Table 1. Mutagenesis assay of PA, DMP and DEP in *Salmonella typhimurium* TA100.

Test compound	μg/plate	$\frac{\text{Revertants per treated plate}}{\text{Revertants per control plate}} \pm \text{SEM}^a$
Hycanthone	30	3.49 ± 0.14
PA	100	1.11 ± 0.07
	300	0.95 ± 0.02
	1000	0.90 ± 0.07
DMP	100	0.82 ± 0.03
	500	1.45 ± 0.10[b]
	1000	1.84 ± 0.12[b]
DEP	100	1.06 ± 0.05
	500	1.26 ± 0.09[b]
	1000	1.90 ± 0.09[b]

[a] The control or background reversion frequency was 291 ± 20 (n = 14). Each value represents the mean from 3-5 replicate experiments. In each experiment duplicate plates were run for each condition.

[b] $p < 0.05$ by Student's *t*-test when tested for difference from 1.00.

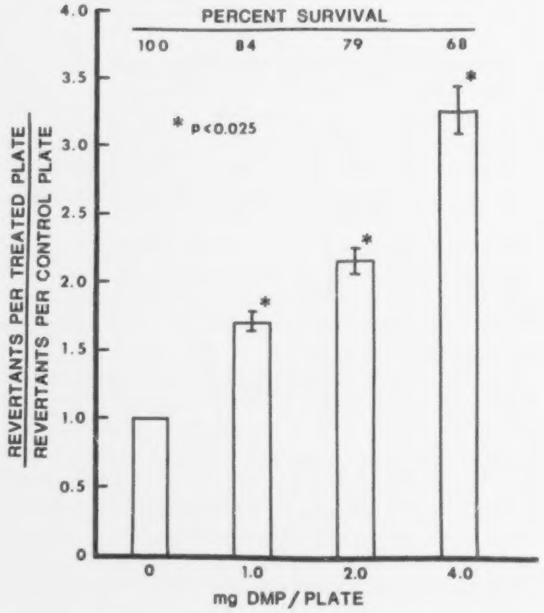


FIGURE 1. Mutagenic dose response for DMP. Each mutagenic value represents a mean from two or more replicate experiments in which duplicate plates (histidine-deficient) were used at each concentration. Percent survival was determined in the following way: known dilutions of TA100 were plated on the nutrient agar (histidine-containing) either in the absence of DMP (control) or in the presence of known amounts of DMP (treated). After 48 hr of incubation, numbers of surviving mutant colonies on the treated plates were compared to control. The asterisk denotes $p < 0.025$ by Student's *t*-test when tested for differences from 1.0.

Table 2. Effect of S-9 supplementation on the Ames assay of DMP and DEP in TA100.

		Revertants per treated plate / Revertant per control plate ± SEM[a]			
			With S9		
Test compound	μg/plate	No S-9	+NADP	+NAD	No c actors
Lucanthone	30	1.04 ± 0.04	2.34 ± 0.13	–	
DMP	1000	1.72 ± 0.14[b]	1.00 ± 0.04	1.03 ± 0.04	1.11 0.04
DEP	1000	1.58 ± 0.21[b]	0.76 ± 0.07	0.69 ± 0.08	0.77 0.08

[a] The control or background reversion frequency was 253 ± 16 (N = 11) in the absence of S-9 and 209 ± 10 in the pr ence of S-9.
[b] p 0.05 by Student's t-test when tested for difference from 1.00.

resented essentially a doubling of the basal level of reversion. These three compounds were not mutagenic when tested with the TA98 strain of Salmonella (data not shown).

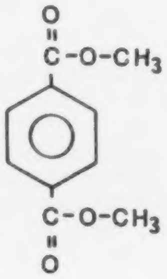
The effect of S-9 liver extract on DMP- and DEP-induced mutations is presented in Table 2. Lucanthone (Luc) was used as the positive control to demonstrate the effectiveness of the S-9 fraction in activating a nondirect-acting mutagen. In the absence of S-9, DMP and DEP produced a positive mutagenic response, confirming the data presented in Table 1. With S-9 and NAD or NADP added to the system, the positive response was reversed. It is important to note that S-9 alone (without the cofactors) also eliminated the mutagenic response.

A number of other phthalate esters were tested in our modified Ames assay. Each phthalate was assayed with bacterial strains TA100 and TA98, both in the presence and absence of S-9 liver extract. All of these phthalate esters failed to produce a positive response at concentrations up to 1000 μg/plate. The compounds tested were: di-*n*-butyl phthalate, di(2-ethylhexyl) phthalate, mono(2-ethylhexyl) phthalate and butyl benzyl phthalate (data not shown).

Figure 1 shows the mutagenic dose response for DMP extended over a broader range of concentrations, i.e., from 1 to 4 mg/plate. A positive dose-related mutagenic response was associated with a dose-related decrease in survival of the cells of the TA100 tester strain. If the absolute number of revertants per plate were corrected for percent survival, an even greater mutagenic response would have been seen than the 3- to 3.5-fold response at 4 mg/plate.

The structure–activity relationship (SAR) of DMP was investigated by testing three other methyl phthalates in the Ames assay with TA100 and TA98, either in the absence or presence of S-9 activation. Dimethyl terephthalate (I), dimethyl isophthalate (II) and trimethyl trimellitate (III) were all negative in the mutagenesis assay.

The results of a host-mediated mutagenic assay of DMP in rats are presented in Figur 2. There were no statistically significant differe ces in the number of revertants per plate for contro or treated



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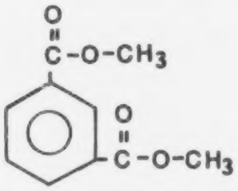

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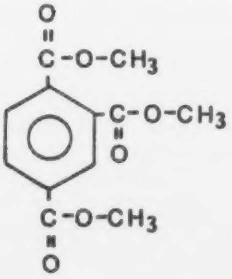

III

urines (over a range of 1 to 8 ml urine) when compared to blank plates. A volume of 8 ml of urine represents approximately 50% of the daily output of control rats and about 30% of the daily output of treated rats. This difference in the fraction of the total daily urinary output represented by a given volume of the two urines was due to a marked diuresis in the DMP-treated rats.

The lack of a mutagenic response to extracts equivalent to up to 8 ml of treated urine (approximately 30% of the daily output) led us to assay the extracts for DMP and other phthalic acid-containing metabolites. These extracts were found to have 0.06 mg/ml of DMP and 1.90 mg/ml of the mono-de-esterified product, monomethyl phthalate (MMP). No other phthalic acid-containing derivatives were found. Thus the mutagenesis plates containing the equivalent of 8 ml of treated urine would have contained 480 µg of DMP and 15.2 mg of MMP. Previous experiments (data not shown) had shown that MMP was not mutagenic in TA100 at levels up to 24 mg per plate.

Since the urinary extracts used in the host-mediated assay were obtained by a single *n*-butanol extraction of urine that was not further acidified beyond its native pH (see methods), further experiments were conducted to determine the phthalic acid-containing components in urine of treated rats using more exhaustive extractions from samples that were acidified to pH 1-2 with HCl. The data are shown in Table 3. DMP and MMP were found at levels of 0.14 and 15.70 mg/ml, representing 0.6 and 67.0% of the injected dose, respectively. Once again, no other phthalic acid-containing derivatives were detected. Thus, DMP represented less than 1% of all phthalic acid-containing molecules excreted in 24 hr.

Although these results suggest systemic inactivation of the mutagenic activity of DMP, it should be recalled that the major route of human exposure is topical. Therefore, the ability of rat skin to metabolize DMP to MMP and thus inactivate the diester was investigated. The results from three separate experiments performed to study the *in vitro* metabolism of DMP by rat skin are presented in Table 4. In the first experiment, only MMP formation was assayed; it was found to be 3.47 nmole/hr/mg tissue. Since the de-esterification of DMP should also result in the formation of methanol, the rate for this product was measured in a second experiment. It was found to be 2.58 nmole/hr/mg tissue. In a third experiment the stoichio-

Table 3. DMP and MMP in the 24-hr urine of rats injected IP with DMP at a dose of 2 g/kg.

Compound	Ester in urine, mg/ml	% of injected dose
MMP	15.70	67.0
DMP	0.14	0.6

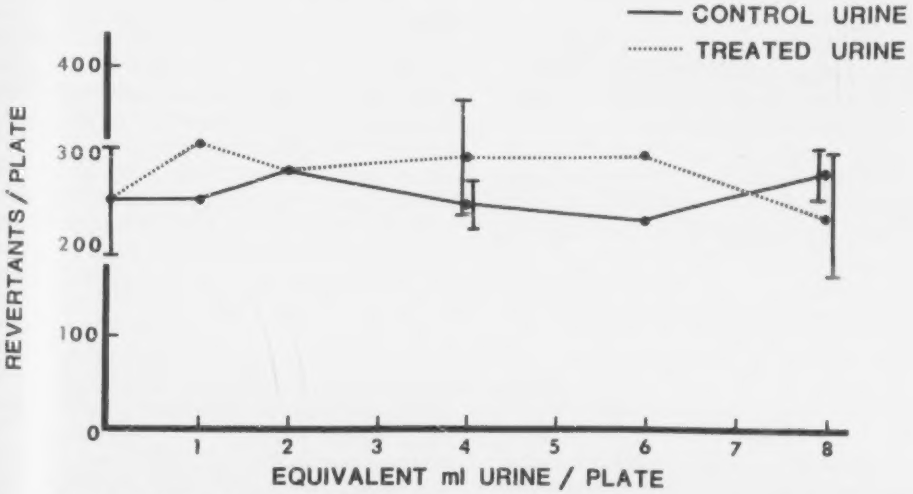

FIGURE 2. Host-mediated mutagenic assay of DMP in rats. Blank plates contained no urine and the data for these are shown on the y-axis. No toxicity (as measured by percent survival on nutrient agar) was seen in TA100 with extracts of either control or treated urine at levels up to 8.0 ml. Data points without standard error bars are the means of values from two independent experiments. Those with standard error bars represent an *n* of 3 or more. Student's *t*-test for these values showed no statistical significance when compared to the data from the blank plates.

Table 4. *In vitro* metabolism of DMP by whole slices of rat skin.[a]

Dimethyl Phthalate → Monomethyl Phthalate + Methanol
(DMP) (MMP)

Exp. no.	MMP formed, nmole/hr/mg tissue (wet)	MeOH formed, nmole/hr/mg tissue (wet)	HCHO formed, nmole/hr/mg tissue (wet)
1	3.47	—	—
2	—	2.58	—
3	2.16	2.18	0

[a] *In vitro* rate of metabolism of DMP by rat liver S-9: 165 nmole MMP formed/hr/mg liver (wet).

Table 5. *In vitro* binding of DMP.[a]

	DMP bound, nmole/mg tissue (TCA precipitable dry weight) $\bar{X} \pm$ SEM
Liver	0.17 ± 0.01
Epidermis	0.98 ± 0.19[b]

[a] Each value represents the mean of four separate assays.

[b] p 0.025 by Student t-test when tested for difference from 0.17 (mean binding value of CMP for liver homogenate).

metric relationship between MMP and methanol was examined and found to be 1:1. The rate of formaldehyde formation was also investigated in this latter experiment, but none of this product could be detected. It should be noted that the rate of de-esterification of DMP by whole skin is approximately 1.5% of the rate seen in the liver, when compared on a mg wet weight basis.

Given the markedly lower rate of metabolism of DMP to a nonmutagenic metabolite in skin relative to liver, the *in vitro* binding of DMP to tissue macromolecules in these two tissues was investigated. The results are shown in Table 5. ^{14}C-Carbonyl-labeled DMP was found to bind to hepatic and epidermal macromolecules at levels of 0.17 and 0.98 nmole/mg tissue (dry weight-TCA precipitable), respectively.

Discussion and Conclusion

The positive dose-related mutagenic response of TA100 to DMP and DEP (Table 1) in the Ames assay was shown to be affected or modified by two factors. One of these is the molecular structure of DMP, as revealed by the SAR study on the effect of position and number of methyl ester moieties on the benzene ring. The failure of the *meta* and *para* dimethyl esters to elicit a positive response in the Ames assay emphasizes the importance of the *ortho* configuration. The addition of a third methyl ester to the 4-position of DMP and resultant elimination of the mutagenic response indicates the necessity for the *ortho* diester arrangement. However, testing of the 1,2,3-trimethyl phthalate is necessary before any conclusive statements can be made in reference to the mutagenic potential of trimethyl esters. Another SAR-related effect is important to note. From the series of phthalate esters tested only the shorter chained and more hydrophilic of the aliphatic phthalate diesters, DMP and DEP, are mutagenic in the bacterial assay.

The elimination of the positive mutagenic response by S-9 liver microsomes (Table 2) implicates metabolism as a second important modifying factor. Lack of involvement of the cofactors NAD and NADP indicates that the classical mixed-function oxidase (MFO) system is not involved in the apparent metabolic elimination of the mutagenic response. Liver S-9 (Table 4) readily metabolizes DMP to MMP, which has been shown to be a nonmutagenic compound in the Ames assay. These results suggest that a S-9 associated esterase activity is responsible for the elimination of the mutagenic effect. Results from the host-mediated mutagenic assay (Fig. 2) support this idea. In this assay approximately 97% of the phthalate ester content of the urine extract is MMP. Thus, the lack of a positive mutagenic response along with the preponderance of monoester indicate the importance of DMP-monoesterase activity in eliminating the mutagenic effect and possibly in affording protection to tissue exposed to DMP.

It should be noted that 8 equivalent ml of treated urine are negative in the mutagenic assay in spite of containing approximately 480 μg of DMP, an amount which results in a statistically significant increase, albeit small, in the mutation rate in the direct assay. It is possible that an interaction with other components of the urine extract might have suppressed the mutagenic response to DMP in the Ames assay. For example, in the extract of 8 ml of urine there was a 33-fold greater level of MMP relative to DMP. This preponderance of monoester could have affected the tester organism interfering with or eliminating its mutagenic response to DMP.

The *in vitro* ability of skin and liver to metabolize DMP was investigated because of the demonstrated importance of metabolism in determining the mutagenic effect of DMP and the recognized fact that these two tissues represent potential mutagenic carcinogenic sites for this compound. When the rates of metabolism of DMP were compared on a wet weight basis, liver had a 60-fold greater rate than whole skin. This marked metabolic difference highlights liver's enhanced capacity to metabolize DMP to a nonmutagenic phthalate form and thus to be protected from a potential mutagenic effect. Conversely, skin, perhaps the only organ exposed to the entire body dose of DMP, may have little inherent metabolic protection against the mutagenic effects of DMP.

In considering the above along with the known ability of many mutagens to bind macromolecules, an investigation of *in vitro* macromolecular binding of ^{14}C-labeled DMP was compared in liver and skin. The results from the binding experiments indicate that DMP binds to epidermal macromolecules at a severalfold greater rate than to liver macromolecules. Thus DMP's lower rate of de-esterification in the skin relative to liver was related inversely to the higher rate of DMP's binding in the epidermis.

In spite of the substantial hepatic metabolism of DMP by rats, the only positive mammalian mutagenic response to this compound was observed by Yurchenko (4) in rat liver. In light of the inverse correlation of binding as well as of mutagenicity to metabolism of DMP, one would have expected the liver to be relatively refractory to a mutagenic event. Thus, based on Yurchenko's observation, either the hepatic metabolism of rats is insufficient to protect the liver from chronic administration of DMP or neither the Ames assay nor the TLC analytical methodology used in these studies is adequate to detect a mutagenic metabolite formed from the products of the mono-esterase activity. If the former explanation prevails, then skin with its considerably lower level of mono-esterase activity might well be at even greater risk than liver to incur a mutagenic/carcinogenic event. On the other hand, if the latter possibility is correct, then the greater rate of hepatic monoesterase activity would suggest liver, rather than skin, as the primary target site.

In conclusion, based on the results presented, we would recommend an *in vitro* bioassay for the carcinogenesis of DMP, particularly in view of the wide spread exposure of the human population to this compound. These results would argue for a bioassay protocol that would include the topical application of DMP.

This work was supported by a training grant (ES07067) and a research grant (ES00454) from the National Institute of Environmental Health Sciences.

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Environmental Health Perspectives
Vol. 45, pp. 111-114, 1982

Mutagenic Activity of Phthalate Esters in Bacterial Liquid Suspension Assays

by John L. Seed*

The mutagenic activities of several phthalate esters have been evaluated in an 8-azaguanine resistance assay in *Salmonella typhimurium*. Three phthalate esters were found to be mutagenic: dimethyl phthalate, diethyl phthalate and di-*n*-butyl phthalate. A number of other phthalate esters were not found to be mutagenic, including di(2-ethylhexyl) phthalate, di-*n*-octyl phthalate, diallyl phthalate, diisobutyl phthalate and diisodecyl phthalate. A metabolite of di(2-ethylhexyl) phthalate, 2-ethylhexanol, was also noted to be mutagenic. The mutagenic activity of this agent and others in this series was dose dependent but weak. No dose-response curve exceeded more than 3.5 times background at maximally testable concentrations. A liquid suspension histidine reversion assay of dimethyl phthalate showed levels of mutagenic activity similar to that observed in the azaguanine resistance assay. The data suggest a need for further investigation of the mutagenic potential of these agents in other assay systems.

Introduction

The phthalate esters are widely used in the manufacture of plastics and are also used for a variety of other commercial purposes. The uses and biological effects of phthalate esters have been described in detail by others in this volume and in previous publications (*1*). Among the adverse biological effects of phthalate esters is an embryotoxic effect reported by Singh et al. (*2*). This increased embryotoxic effect is suggestive of possible mutagenic and carcinogenic activity. In this paper we report on the mutagenic activity of several phthalate esters in 8-azaguanine resistance assays in *S. typhimurium*.

Materials and Methods

Bacteria

Salmonella typhimurium strain TA100 was obtained from Dr. Philip E. Hartman of the Johns Hopkins University.

Phthalate Esters

Ten phthalate esters and analogs were obtained from the NTP repository and were tested for mutagenic activity without further purification. The compounds tested and the commercial sources were, dimethyl phthalate (Aldrich), diethyl phthalate (MRI), di-*n*-butyl phthalate (Aldrich), diisobutyl phthalate (Eastman), diallyl phthalate (Litton Bionetics), di-*n*-octyl phthalate (Kodak), di(2-ethylhexyl) phthalate (MRI), di(2-ethylhexyl) adipate (Pfaltz and Bauer), diisodecyl phthalate (Pfaltz and Bauer) and 2-ethylhexanol (Aldrich). All compounds were dissolved in DMSO (Aldrich, Gold Label) prior to being added to the assay system.

Assays

Bacterial mutagenicity assays were conducted in liquid suspension by using a modification of previously described techniques (*3, 4*). The bacteria were tested for mutation to azaguanine resistance as well as reversion to histidine prototrophy. In azaguanine resistance assays, 10^6 Salmonella organisms were inoculated into a broth containing 2% brain-heart infusion (BHI), 0.1 mg/ml histidine, 0.01 mg/ml biotin and 2% glucose in Vogel-Bonner medium and were incubated 16 hr at 37°C with shaking. The cells were subsequently centrifuged at 1000 *g* for 20 min, resuspended in an equal volume of Vogel-Bonner medium and cooled to 0°C before use. A 50 μl portion of this bacterial suspension (containing 2-4 × 10^9 cells/μl) and 25 ml of test chemical dissolved in DMSO were added to 1 ml of Ca-Mg free phosphate-buffered saline (PBS) pH 7.0. In experiments with S9, 10 μl of S9 and 10 μl of cofactors were also added to the incubation medium. The S9 and the cofactors were prepared as previously described (*5*). The bacteria were exposed to the test chemical for 1 hr in PBS, followed by 3 hr in Vogel-Bonner medium supplemented with 10% BHI and fresh mutagen. In experiments with S9, fresh S9 and cofactors were added as described above. Following exposure to the mutagen, the cells were centrifuged at 1000 *g* for 15 min and resuspended in PBS, pH 7.0. The bacteria were diluted 1:25 and plated on 1.2% Vogel-Bonner agar containing 8-azaguanine and excess citrate as described previously (*3*). The cells were diluted by an additional factor of 2 × 10^4 and plated on Vogel-Bonner agar without azaguanine.

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Histidine reversion assays were conducted essentially as described above with the following modifications. Assay volume was expanded to 5 ml with corresponding increases in the amounts of bacteria and mutagen. Upon termination of the exposure, the cells were centrifuged twice to remove any excess histidine and were resuspended in a volume of 1 ml PBS. Plating of Salmonella (250 μl/plate) was directly on Vogel-Bonner agar containing no histidine. The number of cells plated was determined by diluting 10^6 and plating on agar with excess histidine.

Statistical significance was determined by analysis of variance of log transformed data.

Results

The results of our studies are summarized in Table 1. Only three of the phthalate esters tested were mutagenic: dimethyl phthalate, diethyl phthalate and di-*n*-butyl phthalate. The mutagenic response of these agents in the azaguanine resistance assay is illustrated in Figures 1-3. All of the phthalate esters tested were noted to be weak mutagens. The maximum observed increase in mutant frequency was only 3.5 times background for dimethyl phthalate and 2-ethylhexanol (Fig. 4) and was less for other compounds tested. The maximal concentration tested in these studies was determined by either the limits of solubility in aqueous solution or cytotoxicity exceeding more than 90% of control values. The concentrations required to produce a mutagenic effect ranged from 5 to 10 *mM* dimethyl phthalate to 0.09–0.18 *mM* di-*n*-butyl phthalate. In addition to the weak mutagenic response, the phthalate esters were also noted to be highly cytotoxic at concentrations which were mutagenic. Under the conditions of the assay, absolute increases in mutant frequency were not observed at any concentration tested. However, when increased numbers of cells were plated in order to maintain a concentration of 4 × 10^6 viable cells on the selective plate absolute increases in mutant number were observed which were consistent with the observed increase in mutant frequency seen in Figures 1-3. These observations are consistent with previous observations (J. Seed, unpublished) that mutant yield in this assay is independent of cell density (*3*). The presence of S9 was not important for the detection of mutagenic activity. In fact, S9 inhibited the mutagenic response of the phthalate esters in this assay. Increasing the concentration of S9 fivefold completely blocked the mutagenic response of dimethyl phthalate. The mutagenic response to the phthalate esters was not limited to azaguanine resistance assays. Studies on the mutagenic activity of dimethyl phthalate in liquid suspension histidine reversion assays also demonstrated a dose-related increase in revertant frequency between 5 and 10 *mM* dimethyl phthalate (Fig. 5).

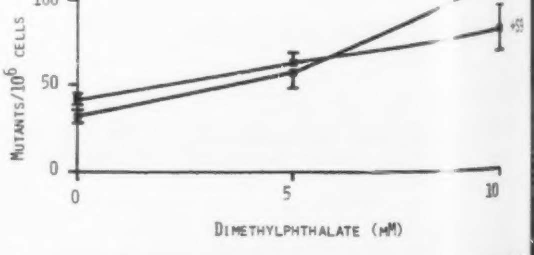

Figure 1. Mutagenic response of Salmonella to dimethyl phthalate in 8-azaguanine resistance assay. Data represent the means of four independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ($F_{3,8} = 37.3$; $p < 0.001$).

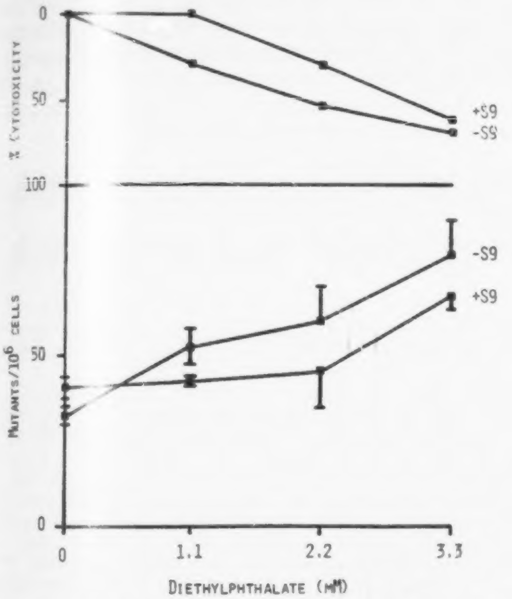

FIGURE 2. Mutagenic response of Salmonella to diethyl phthalate in 8-azaguanine resistance assay. Data represent the means of three independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ($F_{2,9} = 13.7$; $p < 0.005$).

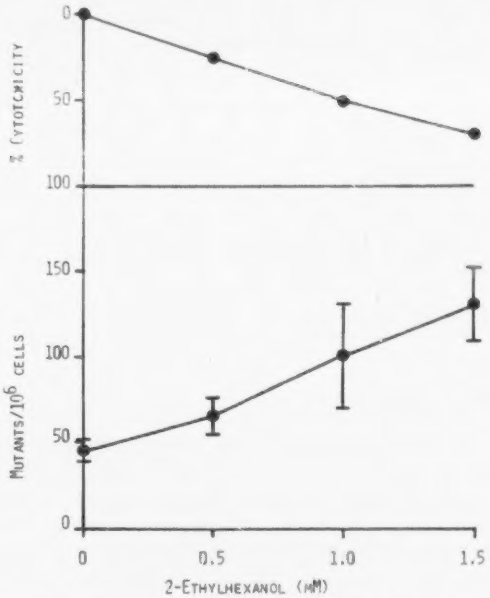

FIGURE 4. Mutagenic response of Salmonella to 2-ethylhexanol in 8-azaguanine resistance assay. Data represent the means of three independent experiments.

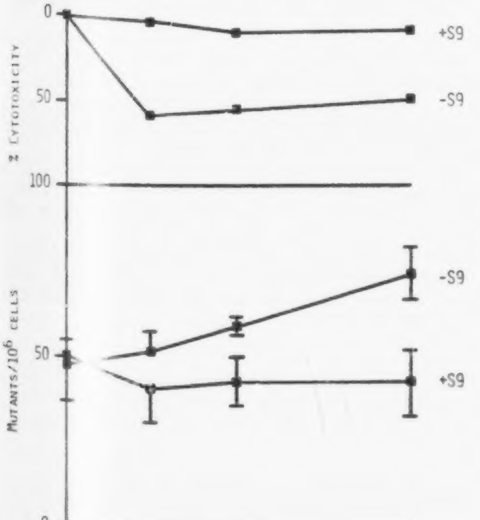

FIGURE 3. Mutagenic response of Salmonella to dibutyl phthalate in 8-azaguanine resistance assay. Data represent the means of three independent experiments. Response was statistically different from control as determined by analysis of variance of transformed data ($F_{2,9} = 6.38$; $p < 0.025$).

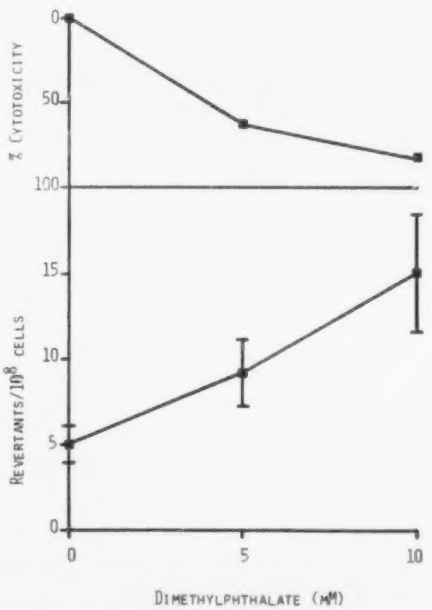

FIGURE 5. Mutagenic response of Salmonella to dimethyl phthalate in histidine reversion assay. Data represent the means of three independent experiments.

Table 1. Phthalate esters and related compounds tested for mutagenic activity in azaguanine resistance assays with *Salmonella typhimurium*.

Compound	Assay result
Dimethyl phthalate	+
Diethyl phthalate	+
Diallyl phthalate	–
Dibutyl phthalate	+
Di-*n*-octyl phthalate	–
Di(2-ethylhexyl) phthalate	–
Di(2-ethylhexyl) adipate	–
2-Ethylhexanol	+
Diisodecyl phthalate	–
Diisobutyl phthalate	–

Discussion

The results of these studies indicate that there may be some mutagenic activity associated with certain phthalate esters. The mutagenic activity which has been observed is, however, very weak. Experiments in our laboratories have failed to detect significant mutagenic activity associated with the phthalate esters in the Ames agar plate assay although others have reported an increase using certain modifications of this test (*6*). Such an observation may not be surprising in view of the generally low levels of mutagenic activity observed. Liquid suspension assays are frequently more sensitive than plate assays (*4*, *7-10*), the increase in sensitivity depending on the compound being tested. In the case of compounds such as the phthalate esters, where significant levels of cytotoxicity are associated with a weak mutagenic response, assays such as the one employed in the current study may be the most reliable indicators of a mutagenic effect.

The fact that certain of the phthalate esters are weak mutagens does not necessarily imply that they are weak carcinogens. It is difficult if not impossible to draw quantitative conclusions on the basis of a bacterial assay alone. In fact, when one is working with such low levels of mutagenic activity as are reported in this paper, it is not certain whether or not there is even a reasonable qualitative correlation between mutagenicity and carcinogenicity. Further studies are required both *in vivo* and *in vitro* before the mutagenic potential of these compounds can be adequately evaluated.

This research was supported by Grant 1-PO1E[illegible]02300 from NIEHS and by contract RP-1436 from EPRI.

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Environmental Health Perspectives
Vol. 45, pp. 115-118, 1982

Antifertility Effects and Dominant Lethal Assays for Mutagenic Effects of DEHP

by John Autian*

Results of past animal studies have indicated the antifertility effects of phthalate esters and additional studies have suggested the potential mutagenic effects at very high doses. A toxicity study in mice has also been conducted in which chronic LD_{50} values were calculated for a group of phthalate esters. For DEHP, for example, the acute LD_{50} was 38.35 ml/kg, but after 10 weeks the value fell to 1.37 ml/kg, suggesting that the ester was producing a cumulative toxicity. A more recent study explored the possibility that DEHP might be a cumulative toxic agent even at lower doses in regard to antifertility and mutagenic effects in mice. Preliminary results indicated that antifertility effects occurred with as little as three subcutaneous doses of 1 ml/kg each. Calculations of mutagenic index revealed a dose-dependent effect but no stoichiometric relationship was established.

Introduction

In the United States, concern for potential health threats from the leaching of phthalate esters used as plasticizers in poly(vinyl chloride) items such as blood bags and tubings dates back to a report published in 1967 by Guess et al. (*1*). Their studies on blood bags containing ACD solution revealed that the plasticizers, as well as degradation products of the ester, were released to the solution. While the quantity of the released chemicals was extremely small, the question was raised for the safety of these products when administered to ill and debilitated patients. Although notice was taken of this report, neither government nor industry took steps to correct the "contaminated" ACD solution or blood products placed in these PVC bags. Their past history of use and their apparent safety mitigated the need for making changes in the plastic though industry had begun to initiate studies to help improve the PVC formula.

Publication of research by Jaeger and Rubin (*2*) in 1970, however, resurrected the plastic's safety into a broader domain. These investigators reported the detection of residual amounts of phthalate esters in a number of organs and tissues of deceased patients who had a recent history of blood therapy from PVC blood bags. From that date, considerable research has taken place on the toxicology of the various phthalates, in particular, di(2-ethylhexyl) phthalate (DEHP). Two major phthalate conferences have also been held; one in 1973 and the present one. Since the initial review of the toxicology of phthalate esters by Autian in 1973 (*3*) additional reviews have appeared in literature. Yet, after a decade and a half, no resolution of the safety issue has occurred, and the volume of medical devices used in medical applications has increased considerably since that time. The recent findings that DEHP has induced tumors in both rats and mice may become the pivotal point for a regulatory agency such as FDA to ban PVC (with phthalate esters) from specific uses (National Cancer Institute, unpublished report).

It is now well established that DEHP and most other diesters of phthalic acid have been found to have a very low order of acute toxicity. Chronic animal studies performed in the 1950s and 1960s (with the exception of the very recent cancer studies) also suggest that low levels of phthalate esters taken in feed on a daily basis have no significant toxicologic consequence, even though there was some indication of liver involvement at the higher dose levels.

A more recent study on chronic toxicity by Lawrence et al. (*4*) permitted the calculations of

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chronic LD_{50} for a group of phthalate esters. In these experiments, a group of mice was injected IP with a series of doses five days per week. At the end of each week, an apparent LD_{50} was calculated. This procedure was continued until the apparent LD_{50} remained constant for three consecutive weeks with a minimum of 10 weeks treatment. Table 1 is a summary of this study and includes the acute LD_{50}, the chronic LD_{50} and a cumulative toxicity factor (acute chronic). It will be noted that there is a dramatic cumulative factor for both the di-*n*-octyl and di(2-ethylhexyl) phthalates as compared to the other phthalates. This suggests that, under the experimental conditions, these dioctyl and di(2-ethylhexyl) phthalates do have a marked cumulative effect. For example, DEHP has an acute LD_{50} of 38.35 ml/kg. Over a 10-week period this value is reduced to 1.37 ml/kg. Presently there is no information which explains this type of response, but it may be that the metabolites of the two esters play a much greater role in the mechanism of action than for the lower molecular diesters; this remains conjecture at this time. It might, however, be pointed out that Kevy et al. (*5*) did find the presence of DEHP (or its metabolites) in livers of monkeys many months after the last administration of the compound. Most investigators, however, do not support the cumulative theory for DEHP based upon their studies in rodents.

Singh et al. (*6*) studied the teratogenic effects of eight phthalate esters in rats and noted that all compounds, to various degrees, induced birth abnormalities and fetal deaths. Esters included the following: dimethyl, dimethoxyethyl, diethyl, dibutyl, diisobutyl, butyl carobutoxymethyl, dioctyl and di(2-ethylhexyl) phthalates. Groups of animals were administered the various esters by IP injections on days 5, 10, and 15 of gestation at several dose levels. The most widely used phthalate, di(2-ethylhexyl) phthalate (DEHP), was administered at two dose levels, 5 ml and 10 ml/kg, but fetal abnormalities were noted only at the higher dose level.

Since PVC blood bags generally contain DEHP as the plasticizer, a study was conducted by Lewandowski et al. (*7*) in which two types of PVC blood bag materials were extracted with rat plasma to give a concentration of DEHP approximately equivalent to the dose a human weighing 60 kg could receive during an exchange transfusion of 21-day-old blood. Several dose levels were used, ranging from 1.4 mg/kg per day to 5.3 mg/kg per day. The various doses were administered to groups of pregnant rats IV from day 6 through day 15 of gestation. No teratogenic effects or adverse consequences were noted in any of the experimental animals as compared to control groups not receiving the DEHP, supporting the contention of the authors that blood products stored in PVC would not present a teratogenic risk to pregnant humans.

In another study by Singh and associates (*8*), DEHP and dimethoxyethyl phthalate (DMEP) were administered by IP injection to adult male mice, and subsequently these mice, individually, were mated to groups of two adult virgin females. This sequence with new females was repeated weekly for 12 weeks. Between days 13 and 17 of gestation, the pregnant mice were sacrificed and the uterine horns and ovaries exposed. Corpora lutea, total number of implantations, early and late fetal deaths, pre-implantation losses and viable fetuses (litter size) were determined. Interpretations of the data suggested to the authors that the two esters had the potential to act as mutagenic agents as well as to suppress fertility.

Recent Preliminary Studies on Antifertility and Mutagenic Effects of DEHP in Mice

As has already been described, DEHP appears to have an antifertility effect when male mice are first treated with the compound and then subsequently mated with virgin female mice. This response in

Table 1. Chronic toxicity of phthalate esters in mice (IP).[a]

Ester	Acute LD_{50} (ml/kg)	Chronic LD_{50} (ml/kg)	Cumulative toxicity factor (acute/chronic)
Dimethyl	3.35	1.18	2.84
Diethyl	2.87	1.39	2.06
Di-*n*-butyl	3.41	0.85	4.01
Diisobutyl	3.84	1.87	2.05
Di-*n*-octyl	67.18	3.09	21.74
Di-2-ethylhexyl	38.35	1.37	27.99
Butyl carobutoxymethyl	6.27	3.04	2.06
Bis(2-methoxyethyl)	3.57	1.41	2.53

[a] From Lawrence et al. (*4*). Information summarized from Table 3 in Lawrence's article. For the chronic study, groups of mice were injected IP with a series of doses five days per week. At the end of each week, an apparent LD_{50} was calculated. This procedure was continued until the apparent LD_{50} remained constant for three consecutive weeks with a minimum of 10 weeks treatment.

general is dose-related. Results from dominant lethal assays also suggest that at high doses DEHP might be acting as a mutagenic agent.

The possibility that DEHP might be a cumulative toxic agent, even at lower doses, prompted our laboratory to initiate several preliminary experiments to clarify whether a possible cumulative effect can impose both an antifertility effect and mutagenic effects during an 8-week period.

Experimental

In the first series of experiments, male mice were injected with DEHP subcutaneously on days 1, 5 and 10 at dose levels ranging from 1.0 ml/kg to 100 ml/kg. Seven or more mice were used for each dose level. Normal virgin females were mated individually with each treated and control male mouse on day 21 following the first injection. Animals of the two sexes were kept together for four consecutive days and females examined every day for the presence of vaginal plugs as an indication of matings. Detection of the vaginal plug was taken as the first day of gestation. Bred females were sacrificed on days 12-13 of gestation, and uterine horns and ovaries were exposed surgically to determine the number of corpora lutea, implantations, preimplantation losses, early fetal deaths and viable fetuses.

A second series of experiments was also conducted essentially following the previously described procedure. Groups of 10 male mice were treated with DEHP subcutaneously on days 1, 5 and 10 at the following dose levels: 1 ml/kg, 2 ml/kg, 5 ml/kg and 10 ml/kg. A group of untreated mice was used as control. Each male mouse was mated with a fresh virgin female 24 hr after the treatment, followed by a change of females every fifth day up to 21 days and then every week up to 8 weeks. All pregnant females were sacrificed as previously and the corpora lutea, implantations, preimplantation losses, early fetal deaths and viable fetuses recorded.

Results

Table 2 summarizes the number of pregnancies occurring for each dose administered, as well as the number of corpora lutea, implants, preimplantation losses, early fetal deaths and live fetuses. It will be

Table 2. Antifertility and mutagenic trends from injection (SC) of DEHP in groups of male mice at days 1, 5 and 10 and mated with normal females on day 21.[a]

Group	Incidence of pregnancy	Number of corpora lutea	Implant	Preimplantation losses	Early fetal deaths	Number of live fetuses
Saline control	14/16 (88) (87.5%)	12.62	10.43	2.19	0.37	10.06
DEHP-treated						
1 ml/kg	5/8 (68) (62.5%)	12.87	7.62	5.25	2.75	4.87
2 ml/kg	3/8 (38) (37.5%)	14.00	5.37	8.63	0.87	4.50
5 ml/kg	3/8 (38) (37.5%)	15.12	4.87	10.25	1.00	3.87
10 ml/kg	3/8 (38) (37.5%)	14.50	4.75	9.75	1.62	3.13
15 ml/kg	2/8 (25)	15.52	3.50	12.02	1.12	2.38
20 ml/kg	0/8 (0)	13.87	—[b]	13.87	—[b]	—[b]
40 ml/kg	1/7 (14) (14/2%)	14.14	1.85	12.29	0.00	1.85
60 ml/kg	0/8 (0)	11.25	—[b]	11.25	—[b]	—[b]
80 ml/kg	1/9 (11) (11.1%)	16.33	1.33	15.00	0.00	1.33
100 ml/kg	0/8 (0)	18.44	—[b]	18.44	—[b]	—[b]

[a] Data are expressed as mean value per animal for each group.
[b] No pregnancies in this group.

Table 3. Mutagenic index (early fetal deaths/total implants per pregnancy) × 100.

Time of mating	Control	DEHP-treated			
		1 ml/kg	2 ml/kg	5 ml/kg	10 ml/kg
Day 2	0.43	1.81	5.0	2.99	3.58
Days 6	0.34	0.52	0.43	1.42	0.66
Days 11	0.66	0.86	2.14	2.64	3.51
Days 16	0.36	2.02	2.50	2.08	1.60
Days 21	0.19	0.80	1.60	2.47	4.44
Weeks 4	0.38	1.40	1.22	1.15	2.69
Weeks 5	0.45	1.82	3.33	1.49	3.48
Weeks 6	0.30	0.76	3.05	2.86	3.33
Weeks 7	0.18	2.35	1.75	1.23	1.31
Weeks 8	0.60	1.17	2.20	1.55	1.68

noted that an antifertility effect is apparent even at the 1 ml/kg dose level, though statistical analysis was not performed on these data. The trend of antifertility increases as the dose administered to the male mice increases. Increased early fetal deaths and preimplantation losses also can be noted with the treated animals, the trend generally increasing as the dose administered is increased. Again, the data are suggestive and not definitive under the experimental conditions employed.

Data generated from the second series of experiments, in which treated male mice were mated with virgin female mice at different intervals (from day 2 to 8 weeks), were used to calculate the mutagenic index (early fetal deaths/total implants × 100). These values are shown in Table 3. A review of the table indicates that the mutagenic index increases as the dose is increased, although again it cannot be said if these increases are significantly different from the control group. The trend, however, is clear; the mutagenic index is higher for treated animals on the average than for the control groups at any of the time periods.

Discussion

Previous studies in this laboratory demonstrated that at high doses (IP), the phthalate esters could act as teratogenic and antifertility agents (*6*). Results from a dominant lethal assay also suggested that the esters might have mutagenic activity (*8*). Since DEHP was found to exert a cumulative toxicity effect in mice, these experiments were undertaken to determine antifertility and mutagenic effects of repeated injection of DEHP in mice at various dose levels.

As suggested in Table 2, antifertility effects were noted with as little as three subcutaneous doses of 1 ml/kg each. Calculations of mutagenic index (early fetal deaths/total implants per pregnancy × 100) have been listed in Table 3 for the various dose levels (1, 2, 5 and 10 ml/kg) over an eight-week period. Even though a dose-dependent effect is apparent, no stoichiometric relationship has been established.

Expression of mutagenic index in terms of per pregnancy excludes any possible antifertility effect from DEHP, although the antifertility effects observed in this study may also be a consequence of the mutagenic effects rendering the sperm incapable of fertilizing the ovum or for the fertilized ovum to develop.

In a parallel study not reported here, the effect of lower doses of DEHP on testicular structure and function did not show any changes in histopathological organization or macromolecular contents (nucleic acids and protein) of the tissue, suggesting that increased fetal deaths are not a consequence of testicular atrophy. Alterations in the activity of certain mitochondrial and lysosomal enzymes of testicular tissue were, however, observed after treatment with DEHP to account for the changes in the functional ability of the reproductive system.

It has always been a point of argument in selecting doses of DEHP and/or the route of administration in experimental animals which simulate conditions close to that of human exposure or which provide predictive toxicologic data. A subcutaneous administration would therefore justify the parenteral administration like IV without any possible direct contact with the organs of concern and would certainly be more practical to assess the toxicity of DEHP than oral route where most of the effects would be due to metabolites rather than DEHP itself. Undoubtedly the doses used in the present study are much higher than would be expected to reach the human system through biomedical devices. However, in view of the marked increase in the chronic toxicity and evident accumulation of DEHP in primates, usage of higher doses of DEHP in such short term studies may help in indicating possible risk of mutagenic effects in human beings exposed to relatively smaller doses over a longer period of time.

Further studies in fertility and mutagenic effects of DEHP and other phthalate esters are continuing and the results will be reported in subsequent publications.

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Environmental Health Perspectives
Vol. 45, pp. 119-125, 1982

Mutagenic/Carcinogenic Potential of DEHP and MEHP

by I. Tomita,* Y. Nakamura,* N. Aoki* and N. Inui†

The mutagenic/carcinogenic activities of DEHP and MEHP were studied in bacteria and mammalian cells. MEHP but not DEHP exerted a dose-dependent DNA damaging effect to *B. subtilis* in Rec-assay. DEHP and MEHP showed mutagenic activities to *S. typhimurium* TA-100, with and without S-9 mix, respectively. MEHP produced not only the mutation in *E. coli* WP2 B/r but also sister chromatid exchanges (SCE) in Chinese hamster V79 cells. It also induced 8AG/6TG-resistant gene mutations and chromosomal aberrations in the V79 cells.

Transplacental administration of DEHP or MEHP to the Syrian golden hamster embryos was carried out by administering DEHP or MEHP to gravid animals on day 11 of gestation, followed by the cultivation of embryonic cells for 15-20 days. Both DEHP and MEHP induced 8AG/6TG-resistant mutation, chromosomal aberrations and morphological transformation in the embryonic cells of the Syrian golden hamster.

Introduction

The mutagenic activity of di(2-ethylhexyl) phthalate, (DEHP) was first reported in dominant lethal experiments of mice by Singh et al. in 1974 (*1*). Since then, several reports concerning the genotoxicity of DEHP on microorganisms and mammalian cells have appeared, though most of the studies failed to demonstrate its genotoxic activities (*2-4*). We have previously presented some evidence that mono(2-ethylhexyl) phthalate (MEHP), a major metabolic intermediate of DEHP in mammals, induced not only embryo/fetotoxic effects on mice but also DNA damaging/mutagenic activity to *Bacillus subtilis* (Rec-assay), *Salmonella typhimurium* TA 100 (reversion plate assay) and *Escherichia coli* WP2 B/r try⁻ (spot test) (*5, 6*).

The present investigation was undertaken to determine whether DEHP and MEHP are mutagenic/carcinogenic in animal cells. The mutagenic/carcinogenic effects of DEHP were examined by using V79 Chinese hamster cells *in vitro* and also by using the embryonic cells of the Syrian golden hamster which were exposed to DEHP transplacentally.

This is the summary of mutagenic/carcinogenic activities of DEHP and MEHP on microorganisms and on hamster cells.

Experimental

Materials

All chemicals, biochemicals and medium constituents were purchased from Wako Pure Chemicals (Tokyo), Tokyo Kasei Chemicals (Tokyo), Sigma Chemical Co. (St. Louis, Mo.), Difco Lab. (Detroit, Mich), or Gibco (Grand Island, N.Y.). All chemicals and biochemicals were of analytical grade. MEHP was synthesized according to the method of Kenyon (*7*) and was more than 99% pure as determined by ECD-GLC analysis. Plastic dishes and flasks for tissue culture were obtained from Falcon Plastic (Oxnard, Calif.).

Assay with bacteria

All chemicals were dissolved in dimethyl sulfoxide (DMSO) for testing.

Tester strains of *S. typhimurium* TA 100, *E. coli* WP2 B/r and *B. subtilis* H17, M45 were provided by T. Kada (The National Institute of Genetics) and T. Matsushima (Institute of Medical Sci., Univ. of Tokyo).

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DNA-damaging test was performed according to Kada's Rec-assay (*8*). The Ames test was conducted by the preincubation method (*9*).

The procedure for the quantitative mutagenicity test was as follows. The bacterial cell suspensions (4×10^9 cells/ml for *E. coli* B/r WP2 and 1×10^9 cells/ml for *S. typhimurium* TA 100) in Dulbecco's phosphate buffered saline were incubated with the MEHP solution for 20 min. After the incubation, the number of revertant colonies was counted. The cytotoxicity was determined by ordinary methods.

Assay with V79 Chinese hamster cells

Mitomycin C (MMC) and ethyl methanesulfonate (EMS) were dissolved in culture medium directly and added to the cells within 30 min. In these cytogenetic studies, the general experimental methods of Ishidate (*3*), Kuroki (*10*) and Perry (*11*) were used.

Assay with Syrian golden hamster

The experimental scheme for *in vivo*/*in vitro* combination assay of the mutation induced by the administration of DEHP/MEHP is as depicted in Figure 1. The application of DEHP and MEHP to Syrian golden hamster, the preparation of primary culture and chromosome, the selection of the mutants and the assay of morphological transformation were all conducted according to the procedure described by Inui (*12*).

Results

Table 1 represents the DNA damaging potential of DEHP and its related compounds in the Rec-assay with *B. subtilis* according to the method of Kada et al. (*8*). The compounds tested were DEHP, MEHP, phthalic acid and 2-ethylhexanol. Differences larger than 1.5 mm in the inhibited zones between H-17 (*rec*$^+$) and M-45 (*rec*$^-$) were observed when 400 or 500 μg of MEHP/disk was used. No differences were observed between the inhibition length of two strains when 500 μg of DEHP or phthalic acid/disk was applied. A slight difference in the inhibitions between the two strains was observed when 500 μg of 2-ethylhexanol/disk was applied. A positive result was obtained when DEHP preincubated with mouse pancreas homogenate was used.

Table 2 shows the mutagenic potential of DEHP and MEHP in the plate assay (*9*) with *S. typhimurium* TA 100 in the presence or absence of S-9 mix. DMSO was used as the control vehicle. Both 4-nitroquinoline-1-oxide (4-NQO) and benzo(a)pyrene were used as reference compounds possessing positive effects. A level of 5 mg of DEHP/plate gave 142 induced revertant colonies/plate with S9 mix, while 1.25 mg of MEHP/plate gave 30 induced revertants/plate without S9 mix. It should be noted, however, that 1.25 mg of MEHP/plate gave no induced revertants in the presence of S9 mix. These results suggest that MEHP itself is an acting principle for the development of mutagenicity and that further metabolism of DEHP by S9 mix would lead to cessation of its activity.

The mutagenic activities of various doses of MEHP to *S. typhimurium* TA 100 and *E. coli* WP2 B/r are shown in Figure 1. The surviving cells of both bacteria decreased with the increased concentration of MEHP, and the resultant numbers of histidine- or tryptophan-independent mutant colonies/surviving bacteria cells increased sharply in a dose dependent manner.

Further studies were performed on MEHP and DEHP with the use of V79 Chinese hamster cells and the embryonic cells of Syrian golden hamster.

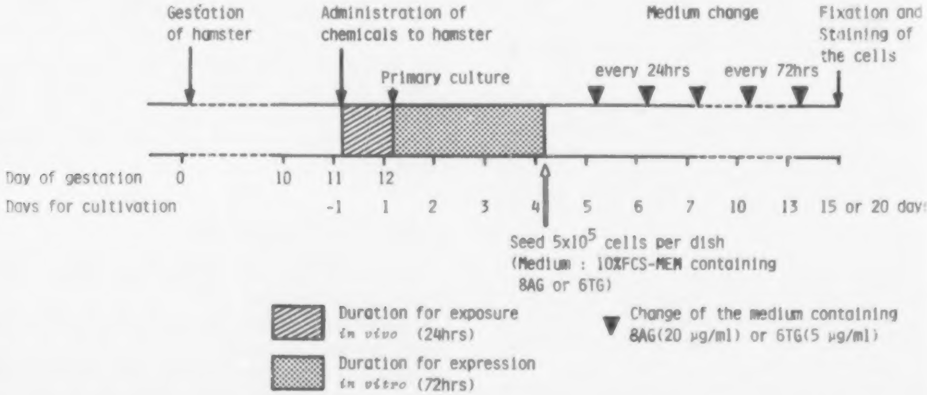
Gestation of hamster
Administration of chemicals to hamster
Primary culture
Medium change
every 24hrs
every 72hrs
Fixation and Staining of the cells
Day of gestation
0
10
11
12
Days for cultivation
-1
1
2
3
4
5
6
7
10
13
15 or 20 days
Seed 5x10⁵ cells per dish
(Medium : 10%FCS-MEM containing 8AG or 6TG)
Duration for exposure in vivo (24hrs)
Duration for expression in vitro (72hrs)
Change of the medium containing 8AG(20 μg/ml) or 6TG(5 μg/ml)

FIGURE 1. *In vivo/in vitro* combination assay procedure with the embryonic cells of Syrian golden hamster

able 1. **DNA-damaging potentials of DEHP and related compounds in Rec-assay with *B. subtilis*.**

		Inhibition zone, mm			
Compounds ed	Concn, μg/disk	H17 (Rec^+)	M45 (Rec^-)	Difference, (mm)	Evaluation
DEHP	500	0	0	0	–
MEHP	50	0	0	0	–
	100	0	0.5	0.5	–
	200	0	0.8	0.8	–
	300	0	1.3	1.3	–
	400	1.3	4.5	3.2	+
	500	1.0	6.0	5.0	+
Phthalic aci	500	0	0	0	–
2-Ethylhexa	500	0.4	1.7	1.3	–

Table 2. M genic potentials of DEHF and MEHP in the plate assay with or without S-9 mix by *S. typhimurium* TA-100.

		Without S9 mix[a]		With S9 mix[a]	
Compounds te ted	Amount plate	Revertant colonies per plate	Induced revertants per plate	Revertant colonies per plate	Induced revertants per plate
DMSO	100 μg	141 ± 7	—	182 ± 22	—
4NQO[a]	0.4 μg	264 ± 22[c]	123		
Benzo(a)pyrene	5 μg			380 ± 50[c]	198
DEHP	5 mg			324 ± 16[c]	142
MEHP	1.25 mg	171 ± 8[b]	30	171 ± 31	0

[a] All experiments were performed with five plates per group.
[b] Significantly different from controls, $p < 0.05$.
[c] Significantly different from controls, $p < 0.01$.

Table 3. Sister chromatid exchanges induced by MEHP in Chinese hamster cells.

Compounds tested[a]	Concentration, μg/ml	Time, hr	Total cells co nted	Total SCEs	Mean SCEs per cell ± SD	Mean SCEs per chromosome ± SD
BrdUrd[b]	2	24	21	105	5.0 ± 1.0	0.24 ± 0.06
MMC[c]	6.7×10^{-3}	24	20	590	29.5 ± 2.2[d]	1.35 ± 0.29[d]
MEHP	25	24	20	146	7.3 ± 2.3[d]	0.36 ± 0.14[e]
	50	24	20	210	10.5 ± 3.4[d]	0.52 ± 0.17[d]
MMC	13.4×10^{-3}	3	27	237	8.8 ± 4.7[e]	0.42 ± 0.25[e]
MEHP	750	3	21	132	6.3 ± 2.6	0.29 ± 0.09
	1000	3	20	124	6.2 ± 2.7	0.30 ± 0.14
	1500	3	20	158	7.9 ± 3.7[e]	0.36 ± 0.17[e]

[a] 2×10^6 c s were seeded per 25 cm^2 T-flask. The cells were cultivated for 22 hr in 10% FCS-MEM containing BrdUrd (2μg/ml) in the presence f chemicals.
[b] 5-Bromo- deoxyuridine.
[c] Mitomyci C.
[d] Significa y different from controls, $p < 0.001$.
[e] Significa y different from controls, $p < 0.01$.

Table 3 ws the results on sister chromatid exchange SCE) induced by MEHP in Chinese hamster c s. An assay was conducted according to the meth of Perry and Wolff (*11*). As shown in Table 3, t cells exposed to 5-bromo-2′-deoxyuridine (BrdUrd) r 24 hr produced 5.0 SCE/cell. The cells treated w h 25 50 μg of MEHP/ml for 24 hr gave 7.3 and 1 5 SCE/cell, respectively, values which were sig icantly higher than the numbers of SCE (5.0) obta ned with BrdUrd only. When the cells were trea ed with 750-1500 μg of MEHP/ml for 3 hr, however, only 1500 μg of MEHP/ml produced a significant increase of SCE/cell. MMC, which was used as a positive reference compound, produced 29.5 and 8.8 SCE/cell on the exposure for 24 hr and 3 hr, respectively.

The study by the *in vivo/in vitro* combination assay system with Syrian golden hamster was conducted according to the method developed by Inui (*12*). As shown in Figure 2, 0.5 ml of DMSO containing DEHP or MEHP was administered orally to gravid Syrian golden hamsters on the 11th day of gestation and the animals were kept for 24 hr with free access of laboratory chow and water. The fetuses were then excised and used for primary culture for 3 days. The cells (5×10^5) were then transferred to dishes containing 8-azaguanine (8AG) or 6-thioguanine (6TG) in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) in order to examine the induction of the *HGPRT*⁻ forward mutation. The medium was changed every 24 hr for the first three days and then once every 72 hr for the remaining time.

After a total cultivation period of 15 to 20 days, the cultures were fixed in methanol and stained with Giemsa solution. The number of colonies growing in the medium was counted. Table 4 summarizes the data on chromosomal aberrations induced by the transplacental application of DEHP and MEHP to the embryonic cells of the Syrian golden hamster. The range of the dosage tested was from 3.75 g to 15 g/kg in DEHP and from 0.375 g to 1.5 g/kg in the case of MEHP. As seen in Table 4, 3% of the cells were aberrant in the control experiment, whereas from 10% to 24% of the cells were aberrant with DEHP or MEHP. The types of aberrations include a single chromatid gap, isochromatid gap, a single chromatid break, isochromatid break, chromatid exchanges and other aberrations including chromosome fragmentations and duplications, etc. The morphological transformations of the embryonic cells of the Syrian golden hamster are shown in Table 5.

Cells (5×10^3) of the hamster embryos receiving DEHP or MEHP transplacentally were cultured in 20% FCS-MEM for 7-8 days in plastic dishes (60 mm in diameter) without changing the medium. After fixing and staining with Giemsa solution, the total number of colonies, the number of transformed colonies and the types of colonies were examined. The types of colonies were classified a cording to Heiderberger et al. (*13*). The transfor ation rate was expressed as the percentage of mor pologically transformed colonies against the num ers of the survived cells. In this experiment, the ells of the hamster embryos receiving *N*-nitrosodir thylamine

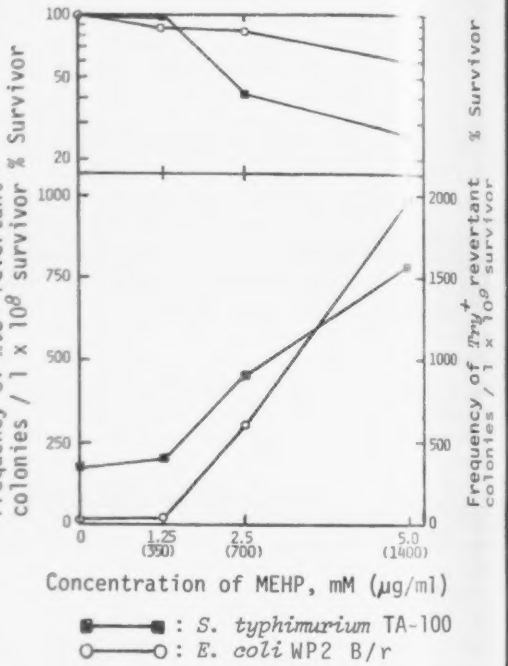

FIGURE 2. Mutagenic activities of MEHP to *S. phimurium* TA 100 and *E. coli* WP2 Br.

Table 4. Chromosomal aberrations induced by the transplacental application of DEHP and MEHP in embryonic c ls of Syrian golden hamster.

Compounds tested	Dose of chemicals, mg/kg	Type of aberration[a] SG	IG	SB	IB	E	Other	Aberrant metaphase cells, %[b]	Normal iploid cells % nge)[c]
DEHP	0	0	0	2	0	0	1	3	55 (2 18)
	3750	3	0	1	2	0	2	8	43 (1 17)
	7500	4	1	3	1	0	5	10[e]	40 (3 70)
	15000	6	3	12	0	4	3	24[f]	70 (3 58)
MEHP	375	6	3	5	0	0	3	14[e]	67 (3 52)
	750	9	1	4	1	0	1	14[e]	66 (3 18)
	1500[d]	5	1	6	2	2	2	22[f]	74 (4 16)

[a] Aberrations: SG, single chromatid gap; IG, isochromatid gap; SB, single chromatid break; IB, isochromatid break; exchange; Other, others including ring formation, fragmentation and other chromosomal aberrations.

[b] 100 metaphase cells examined.

[c] Percentage of normal diploid cells ($2n = 44$) with show or without chromosomal gaps, breaks or other small abnor lities. The values in parentheses the range of the number of chromosomes.

[d] Only 50 metaphase cells examined.

[e] Significantly different from controls, $p < 0.05$.

[f] Significantly different from controls, $p < 0.001$.

(NDMA) transplacentally were used as a positive control.

As seen from Table 5, the transformation ratio was significantly high in the cells treated with 7500 or 15,000 mg of DEHP/kg and with 375 or 750 mg of MEHP/kg of the body weight of the Syrian golden hamster. Figure 3 shows the microscopic appearance of the cells of morphologically transformed colonies. Figure 3*a* shows normal colonies; Figures 3*b* and 3*c* represent typical transformed colonies of Type II and Type III, respectively, induced by DEHP treatment. Figure 3*d*, which is an enlargement of Figure 3*c*, shows a criss-cross and dense piling up in the center of the cells.

Discussion

The results of our present investigation suggest that DEHP itself is not a mutagen but becomes a mutagen of base pair substitution type after it is hydrolyzed into MEHP. The mutagenic activity of MEHP which was observed in both Rec-assay and reversion plate assay were increased in a dose-related manner. It must be noted, however, that the demonstration of the mutagenicity of MEHP can only be possible within a high and narrow range of its concentration because MEHP has a sterilizing effect at high concentrations and shows no mutagenic activity at low concentrations. Moreover, the activity of MEHP can be completely destroyed by incubating

Table 5. Morphological transformation in embryonic cells of Syrian golden hamster induced by the transplacental application of DEHP and MEHP.

Compounds administered	Dose of chemicals, mg/kg	No. of animals used	Total observed colonies	Transformed colonies	Type of colony[a]		Transformation rate, %[b]
					II	III	
DEHP	0	6	4359	20	11	9	0.46
	3750	5	2557	20	15	5	0.78
	7500	7	3155	49	21	28	1.55[c]
	15000	7	2549	46	22	24	1.80[c]
MEHP	375	5	2537	27	15	12	1.06[d]
	750	6	1913	24	13	11	1.25[e]
	1500	6	1463	13	7	6	0.89
NDMA[f]	200	1	364	14	9	5	3.85[c]

[a] Colony types classified according to Heiderberger et al. (*13*).

[b] Transformation rate is expressed as the percentage of morphologically transformed colonies against the total survivors.

[c] Significantly different from controls, $p < 0.001$.

[d] Significantly different from controls, $p < 0.05$.

[e] Significantly different from controls, $p < 0.01$.

[f] *N*-Nitrosodimethylamine.

Table 6. Summary of the mutagenic and carcinogenic potency of DEHP and MEHP.

Method of testing	Results	
	DEHP	MEHP
Bacterial systems (*in vitro*)		
Rec-assay		
B. subtilis H17, M45	− (+)[a]	+
Reverse mutation		
E. coli WP2	−	+
S. typhimurium TA 98	−	−
S. typhimurium TA 100	− (+)[b]	+ (−)[b]
Mammalian systems		
Direct application (*in vitro*), V79 cells of Chinese hamster		
8AG/6TG-resistant mutation	NP[c]	± (±)[b]
Chromosomal aberration	NP	+
Sister chromatid exchange	NP	+
Transplacental application (*in vivo*/*in vitro*) to embryonic cells of Syrian golden hamster		
8AG/6TG-resistant mutation	±	±
Chromosomal aberration	+	+
Morphological transformation (colony assay)	+	+

[a] Mouse pancreas homogenate was used for the determination of mutagenicity.

[b] Rat liver microsome fraction was used for the determination of mutagenicity.

[c] Not performed.

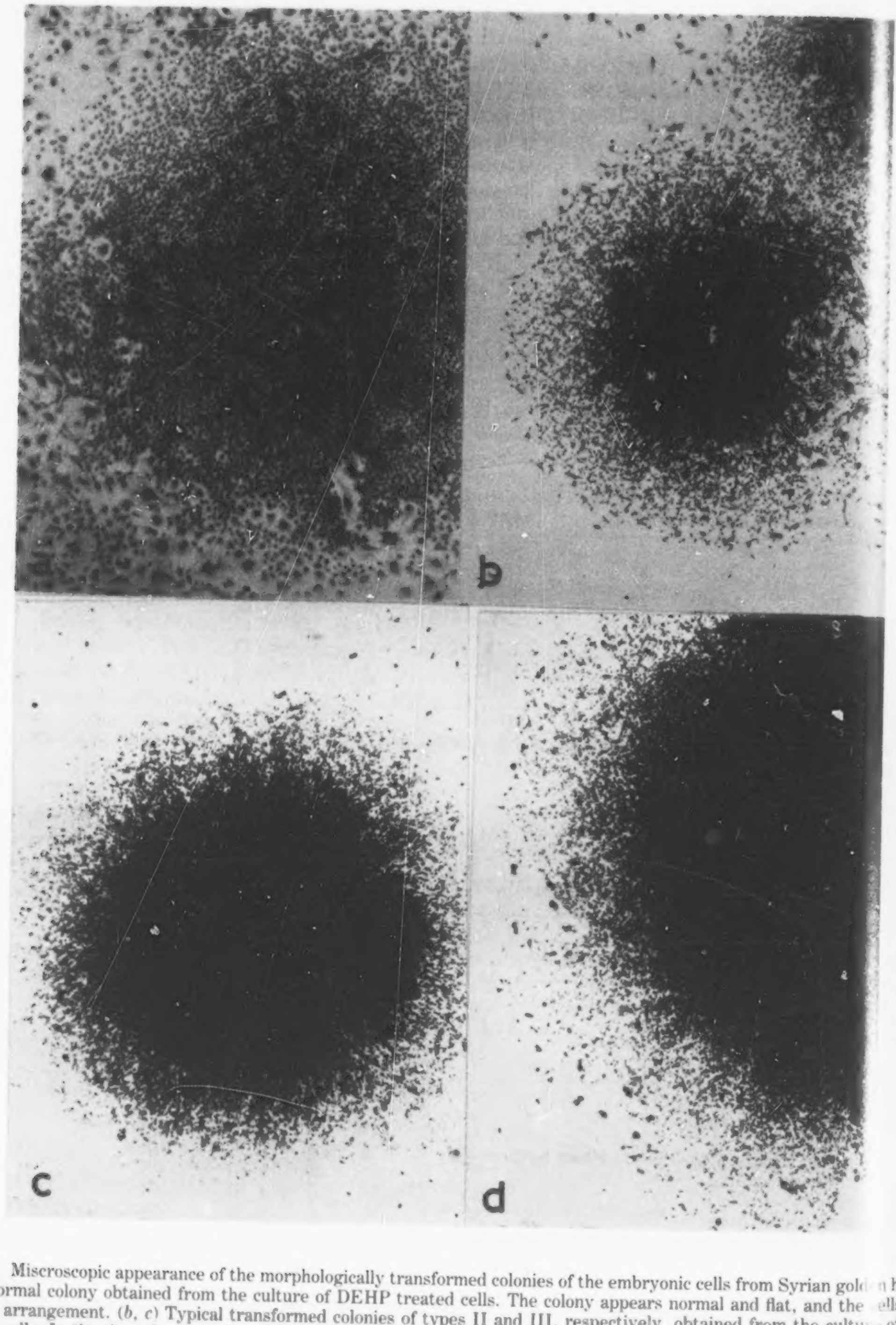

FIGURE 3. Miscroscopic appearance of the morphologically transformed colonies of the embryonic cells from Syrian golden hamster. (a) A normal colony obtained from the culture of DEHP treated cells. The colony appears normal and flat, and the cells have a regular arrangement. (*b*, *c*) Typical transformed colonies of types II and III, respectively, obtained from the culture of DEHP treated cells. In (*b*), the colony consists of short settled fibroplastic cells with massive piling up into virtually opaque multilayers and the cells are only moderately polar. In (*c*), the colony is composed of highly polar, fibroplastic multilayered criss-crossed arrays of densely stained cells. (*d*) Enlargement of Fig. 3*c*.

it with the S9 mix. It is, possibly, for this reason, that the mutagenesis of DEHP (with the S9 mix) and MEHP have not always been demonstrated. As shown in Table 3, SCEs in Chinese hamster V79 cells were induced by MEHP, even at the level of 25 μg/ml in the 24 hr incubation study. As the induction of SCE is believed to be a sensitive reflection of the toxicities related to DNA repair (*14*), it is conceivable that MEHP is the substance having the DNA-damaging effect in Rec-assay and induces SCEs. It was also demonstrated that MEHP induced 8AG- or 6TG-resistant gene mutations and chromosomal aberrations in Chinese hamster V79 cells *in vitro*, though the details of the results are not included in this paper. The effects of DEHP on V79 Chinese hamster cells have not been studied in our laboratory, but negative results will probably be observed unless the conditions are so chosen as to yield enough MEHP during the incubation.

As for the genotoxic effects of DEHP on the embryonic cells of Syrian golden hamster, it will be reasonable to assume that the effects will be due to MEHP formed *in vivo* in the hamster. In fact, 3.9 μg of MEHP/g of fetuses was formed when 10 ml of DEHP/kg was administered orally to gravid Syrian golden hamster at 11-12 days of gestation (our unpublished results). Among several effects of DEHP and MEHP on the embryonic cells of Syrian golden hamster, the positive results on the morphological transformation should be carefully considered, since the cell transformation is closely correlated with carcinogenicity (*13*). The results of our studies on DEHP and MEHP mutagenesis/carcinogenesis are summarized in Table 6.

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Commentary

Environmental Health Perspectives
Vol. 45, pp. 127-128, 1982

An Overview of Phthalate Ester Carcinogenicity Testing Results: The Past

by J. Wilbourn* and R. Montesano*

This paper presents a short review of the available carcinogenicity tests which have been carried out on various phthalate esters prior to the availability of the National Cancer Institute bioassay studies. All of these studies suffer from diverse limitations in design or reporting when compared to more recent experimental protocols and reports.

There are three papers related to di(2-ethylhexyl) phthalate, one to di-*n*-butyl phthalate, two to butyl benzyl phthalate, one to ethyl phthalyl ethyl glycolate and another to butyl phthalylbutyl glycolate (*1*).

Di(2-ethylhexyl) Phthalate (DEHP)

The first experiment was carried out in 1953 in Sherman rats. Groups of 32 male and 32 female rats were administered 0.04, 0.13 or 0.4% DEHP in the diet for a maximum of two years. The doses were equivalent to 0. 20, 60 and 200 mg/kg body weight/day, respectively. Controls (number unspecified) received basal diet alone. After one year, 42% of the control rats and the same percentage of rats at the highest dose level survived. The various groups were reduced to a maximum of eight males and eight females per group at the end of one year. Two groups of 32 males and 32 females (F_1 progeny) were generated from the control and high dose P generation and at 15 days of age were maintained at a dose level of 0.4% DEHP in the diet for one year, at which time the animals were killed. Some tumors were observed in the animals surviving longer than a year, but they were equally scattered among the different groups, so that, on the basis of this experiment, it was not possible to conclude either a negative or positive carcinogenic response to this chemical (2). The small number of animals, poor survival and limited reporting make this experiment inadequate in terms of a carcinogenicity bioassay.

The second experiment was carried out by Harris et al. (*3*) on Wistar rats. Groups of 43 males and 43 female rats were fed DEHP at a dose of 0, 0.1% and 0.5% in the diet for a maximum of two years, which corresponded to an intake of about 0, 70 and 250 mg/kg body weight/day, respectively. In this experiment, groups of animals were sacrificed at various times, namely, at 3, 6, 12, and 24 months, the number of animals sacrificed being four, four, 10 and 24 rats, respectively. Pathological examination, however, was not carried out for all animals sacrificed, but only in eight males and eight females per group at 12 months, and in one to a maximum of four rats in the treated and control groups at two years. No tumors were observed. In addition to the limited pathology examination carried out, this study has the same limitations as the previous experiment in regard to the possibility of assessing the carcinogenicity of this chemical.

An experiment in 1974 by Onda et al. cited by Omori (*4*) which was originally reported as an abstract in Japanese, mentions the induction of renal cysts in the F_1 and F_2 generations of ddY or ICR mice treated with 10 or 100 mg/kg body weight/day DEHP, but it is not clear if the treatment of the animals was carried out only in the parent generation, or if it was continued also into F_1 and F_2 generations. This experiment could not be evaluated for carcinogenicity.

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Di-*n*-butyl Phthalate

A similar experiment cited by Omori (*4*) on di-*n*-butyl phthalate reported less severe effects on the kidney; this study also cannot be used for an evaluation of carcinogenicity.

Butyl Benzyl Phthalate

An experiment was carried out on butyl benzyl phthalate by Theiss et al. (*5*) on male strain A/St mice aged 6-8 weeks, in which mice were given IP injections of 160, 400 or 800 mg/kg body weight three times/week for 8 weeks. The animals were killed at 24 weeks and examined for the number of lung tumors detected macroscopically. The numbers of lung tumors per mouse were 0.1-0.25 in treated groups, compared with 0.39 in the corresponding vehicle controls. A negative effect in this test system cannot be taken as evidence for the absence of a carcinogenic effect.

Butyl benzyl phthalate was administered to a group of 20 male and 20 female Wistar rats at a dose of 5 mg/kg body weight/day (exact route and length of treatment unspecified). There was 100% survival at two years. Nontumorous pathological changes were reported to occur in the kidney as well as atrophy of the testes, but no tumors were observed. No details were given concerning control rats, which makes this experiment (*6*) difficult to evaluate.

Ethyl Phthalylethyl Glycolate

Ethyl phthalylethyl glycolate was administered to Wistar rats at doses of 0.05, 0.5 and 5% in the diet for a period of two years. No difference in survival occurred between the two lowest dose groups and the controls (exact survival times not reported), whereas none of the animals in the 5% dose group survived longer than 72 weeks. In this experiment, no tumors were observed, but some kidney pathology was observed in the highest dose group (*7*). The lack of details on survival makes this experiment impossible to evaluate.

Butyl Phthalylbutyl Glycolate

Butyl phthalylbutyl glycolate was administered to groups of 20 Sherman rats (sex unspecified) at three dose levels (200, 2000 and 20,000 mg/kg body weight) in the diet for two years. A group of 40 controls received the basal diet. Although no tumors were reported to occur, more than 80 rats were killed before the end of the observation period, and it is difficult to assess the number of animals surviving or at risk to develop tumors (*8*).

In conclusion, these various experiments do not allow any evaluation of the carcinogenicity of these chemicals in mice or rats.

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Environmental Health Perspectives
Vol. 45, pp. 129-133, 1982

Carcinogenicity Testing of Phthalate Esters and Related Compounds by the National Toxicology Program and the National Cancer Institute

by W. M. Kluwe,[*] E. E. McConnell,[*] J. E. Huff,[*] J. K. Haseman,[*] J. F. Douglas[†] and W. V. Hartwell[†]

Five phthalate esters and related compounds (phthalic anhydride, phthalamide, di(2-ethylhexyl) phthalate, di(2-ethylhexyl) adipate and butyl benzyl phthalate) have been tested for carcinogenic effects in standard lifetime rodent feeding studies. Groups of 50 male and female rats and mice were fed diets containing various concentrations of the test chemicals for 102–106 consecutive weeks. The dietary concentrations were estimated to be maximally tolerated doses and half maximally tolerated doses. All animals that died during the study and all survivors at the end of two years were examined grossly and microscopically for the presence of tumors. The incidences of animals with tumors at a specific anatomic site in the treated groups and the controls were compared statistically.

Neither phthalamide nor phthalic anhydride increased tumor incidences in rats or mice. Di(2-ethylhexyl) phthalate increased the incidences of liver tumors in rats and mice of both sexes, while di(2-ethylhexyl) adipate caused liver tumors in male and female mice, only. Butyl benzyl phthalate did not cause tumors in male or female mice, but the incidence of myelomonocytic leukemia in butyl benzyl phthalate-treated female rats was significantly greater than that in the controls. Chemically induced early deaths in the butyl benzyl phthalate-treated male rats precluded an evaluation of carcinogenic potential in this sex.

Under the conditions of these tests, di(2-ethylhexyl) phthalate was considered to be carcinogenic in both rats and mice and di(2-ethylhexyl) adipate was considered to be carcinogenic in mice. The evidence for carcinogenic effects of butyl benzyl phthalate in female rats was judged to be equivocal because of the variable nature of the incidence of myelomonocytic leukemia in Fischer 344 rats. Phthalamide and phthalic anhydride did not exhibit carcinogenic effects in these studies.

Introduction

Di(2-ethylhexyl) phthalate (DEHP), di(2-ethylhexyl) adipate (DEHA) and butyl benzyl phthalate (BBP) are chemicals frequently added to plastic formulations to impart flexibility or other characteristics that determine product use. Such plasticizers do not become part of the polymer matrix and, under certain use or disposal conditions, can migrate from the plastic to the external environment. Although they are not components of the plastic polymer, plasticizers such as DEHP may comprise as much as 50% (by weight) of highly flexible poly(vinyl chloride) products. DEHP is also used as a hydraulic fluid and as a dielectric fluid. Phthalate esters, not surprisingly, have become ubiquitous environmental pollutants.

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Phthalate esters are generally synthesized by the condensation of alcohols with phthalic anhydride. Phthalamide is not used as a plasticizer or in the synthesis of phthalate esters. Rather, it is an intermediate in the commercial synthesis of organic amines. All five of these chemicals are being compared, however, because of similarities in chemical structure (Fig. 1).

The National Toxicology Program (NTP) and its predecessor, the National Cancer Institute Carcinogenicity Bioassay Program, tests chemicals for carcinogenic potential as part of a broad initiative to aid governmental regulatory agencies in protecting the public health. Among the criteria used in choosing chemicals for testing are volume of production, number of persons exposed and frequency and level of anticipated human exposure. Phthalate esters and related compounds were tested because of their presence in high concentrations in plastics, the ubiquity of phthalate ester-plasticized products in the home, work and out-of-doors environments and because of a general lack of previous chronic toxicity testing with these agents.

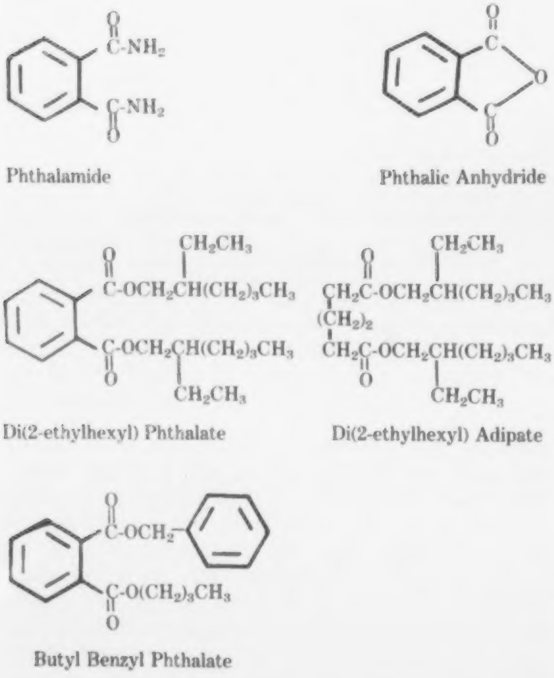

FIGURE 1. Chemical structures of the five compounds tested.

Methods

Male and female rats of the inbred Fischer 344 strain and male and female mice of the hybrid B6C3F$_1$ strain, obtained from Frederick Cancer Research Center (Frederick, Md.), were used in all of the studies. The test chemicals were incorporated into the feed and supplied to the animals for 102–106 consecutive weeks beginning at -6 weeks of age. In general, each treatment group consisted of 50 animals of each sex and species at the estimated maximally tolerated dose (referred to as the high dose), one-half of the estimated maximally tolerated dose (referred to as the low dose) and untreated controls (control groups for phthalamide and phthalic anhydride consisted of 20 animals, control groups for other chemicals consisted of 50 animals). Estimated maximally tolerated doses were determined by preceeding 90-day subchronic feeding studies.

All animals that died during the study (and were not excessively cannibalized or autolyzed) and all survivors at the end of the study were subjected to a gross necropsy and a complete micropathological examination. Statistical comparisons of the incidences of animals with tumors at a specific anatomical site and of survival and body weight gain were made using both pairwise comparisons and trend tests. The basic designs of the studies conformed to the recommendations of the National Cancer Institute (*1*). Complete technical details of each of the bioassays are available in the form of technical reports from the NTP (*2-6*). Test chemical source, composition and doses for each of the studies were as follows.

Phthalamide

Phthalamide was obtained from Sherwin-Williams, Inc. (Pittsburgh, Pa.) and demonstrated by HPLC to be greater than 99% pure. Concentrations of phthalamide in the feed were 0 (control), 1[illegible],000 and 30,000 ppm for male rats; 0, 5,000 and 10,000 ppm for female rats; 0, 25,000 and 50,000 ppm for male mice; and 0, 6,200, 12,500 and 25,000 ppm for female mice.

Phthalic Anhydride

Phthalic anhydride was obtained from Koppers Co. (Pittsburgh, Pa.) and demonstrated by HPLC to be 98.8% pure. Concentrations of phthalic anhydride in the feed were 0 (control), 7,500 and 15,000 ppm for male and female rats. Male mice were fed diets containing 0, 25,000 and 50,000 ppm for the initial 32 weeks and 0, 12,500 and 25,000 ppm for the remainder of the study; female mice received 0, 25,000 and 50,000 ppm for 32 weeks and then 0, 6,250 and 2,500 ppm for the rest of the study.

Di(2-ethylhexyl) Phthalate

DEHP was obtained from W. R. Grace and Co. (Fords, N.J.) and demonstrated by thin-layer and vapor-phase chromatography to be greater than 99.5% pure. Concentrations of di(2-ethylhexyl) phthalate in the feed were 0 (control), 6,000 and 12,000 ppm for male and female rats and 0, 3,000 and 6,000 ppm for male and female mice.

Di(2-ethylhexyl) Adipate

Di(2-ethylhexyl)adipate was obtained from W. R. Grace and Co. (Fords, N.J.) and demonstrated by thin-layer and vapor-phase chromatography to be greater than 98% pure. Concentrations of di(2-ethylhexyl) adipate in the feed were 0 (control), 12,500 and 25,000 ppm for both male and female rats and mice.

Butyl Benzyl Phthalate

Butyl benzyl phthalate was obtained from Missouri Solvents and Chemicals (Kansas City, Mo.) and demonstrated by thin-layer and vapor-phase chromatography to be greater than 98% pure. Concentrations of butyl benzyl phthalate in the feed were 0 (control), 6,000 and 12,000 ppm for both male and female rats and mice.

Results

Phthalamide

Phthalamide administration did not decrease body weight gain nor lessen the probability of survival in either sex of rats or male mice. Survival for female mice at the end of two years, however, was only 36% in the high dose group in comparison to 80% for the controls. The incidences of phthalamide-treated animals with tumors at a specific anatomical site did not differ significantly from those of the controls.

Phthalic Anhydride

Body weight gains in male rats and in both sexes of mice were reduced by phthalic anhydride, but survival in all of the treated groups of animals was unaffected. The incidences of phthalic anhydride-treated animals with tumors at a specific anatomical site did not differ significantly from those of the controls.

Di(2-ethylhexyl) Phthalate (DEHP)

DEHP treatment decreased body weight gains for female mice and for male and female rats. Animal survival, however, was not compromised by dietary ingestion of DEHP. Liver tumors occurred in both DEHP-treated rats and mice at incidences significantly greater than in the respective controls (Table 1). The incidences of animals with hepatocellular carcinomas was significantly greater for female rats and for male and female mice treated with DEHP than for the controls.

Table 1. Incidences of animals with liver tumors in the carcinogenicity bioassay of di(2-ethylhexyl) phthalate.[a]

Species	Sex	Liver tumor	Incidence		
			Control	Low dose	High dose
Rat	Male	Hepatocellular carcinoma	1/50	1/49	5/49
		Neoplastic nodule	2/50	5/49	7/49
		Hepatocelluiar carcinoma or neoplastic nodule	3/50	6/49	12/49[b]
Rat	Female	Hepatocellular carcinoma	0/50	2/49	8/50[c]
		Neoplastic nodule	0/50	4/49	5/50[b]
		Hepatocellular carcinoma or neoplastic nodule	0/50	6/49[b]	13/50[d]
Mouse	Male	Hepatocellular carcinoma	9/50	14/48	19/50[b]
		Hepatocellular adenoma	6/50	11/48	10/50
		Hepatocellular carcinoma or adenoma	14/50	25/48[b]	29/50[c]
Mouse	Male	Hepatocellular carcinoma	0/50	7/50[b]	17/50[d]
		Hepatocellular adenoma	1/50	5/50	1/50
		Hepatocellular carcinoma or adenoma	1/50	12/50[b]	18/50[d]

[a] Fischer 44 rats and B6C3F$_1$ mice were fed diets containing 0 (control), 3,000 (low dose mice), 6,000 (high dose mice, low dose rats) or 12,000 ppm (high dose rats) of DEHP for approximately two years. The ratios of animals bearing liver tumors to the total number of animals examined microscopically are depicted.

[b] Significantly greater than controls, $p < 0.05$.

[c] Significantly greater than controls, $p < 0.005$.

[d] Significantly greater than controls, $p < 0.001$.

Di(2-ethylhexyl) Adipate (DEHA)

Ingestion of DEHA decreased body weight gains in male and female rats and mice. Animal survival, however, was not compromised. The incidences of tumors at specific anatomical sites in DEHA-treated rats were not increased relative to the controls, but liver tumors (hepatocellular carcinomas in female mice, hepatocellular adenomas in male mice) occurred more frequently in DEHA-treated mice than in the controls (Table 2).

Butyl Benzyl Phthalate (BBP)

Body weight gains in male and female mice and in female rats were reduced by the ingestion of BBP, but survival was unaffected. In contrast, excessive numbers of BBP-treated male rats died from apparent internal hemorrhaging after approximately 3 months of exposure. The study in male rats was terminated early, therefore, and no evaluation of tumorigenic response in the male rats could be made. The incidences of tumors at specific anatomical sites in BBP-treated male and female mice did not differ significantly from those in controls. The incidence of animals with myelomonocytic leukemia, however, was greater for the high-dose female rats than for the controls (Table 3).

Discussion

Neither phthalamide nor phthalic anhydride was demonstrated to be carcinogenic for Fischer 344 rats or $B6C3F_1$ hybrid mice, under the conditions of the standard bioassay, since the incidences of chemically treated animals with tumors at a specific anatomical site did not differ significantly from those in controls.

DEHP was considered to be carcinogenic in both Fischer 344 rats and $B6C3F_1$ mice because of the significantly increased incidences in test chemical-treated animals of hepatocellular carcinomas in male and female mice and in female rats. The incidence of male rats with either hepatocellular carcinomas or neoplastic nodules (total liver tumors) was also significantly greater in the high-dose group than in the controls. The absence of nonneoplastic lesions in the liver and the failure of DEHP to reduce survival suggests that the liver tumors were not caused by an indirect hepatotoxic effect.

DEHA was considered to be carcinogenic in $B6C3F_1$ mice because of the greater incidence in test chemical-treated animals of hepatocellular carcinomas in female mice. Since only the incidence of hepatocellular adenomas (benign liver tumors), not that of hepatocellular carcinomas (malignant liver tumors), was significantly increased in the male

Table 2. Incidences of mice with liver tumors in the carcinogenicity bioassay of di(2-ethylhexyl) adipate.[a]

Species	Liver tumor	Incidence		
		Control	Low dose	High dose
Male	Hepatocellular carcinoma	7/50	12/49	[illegible]/49
	Hepatocellular adenoma	6/50	8/49	[illegible]/49[b]
	Hepatocellular carcinoma or adenoma	13/50	20/49	[illegible]/49[c]
Female	Hepatocellular carcinoma	1/50	14/50[d]	[illegible]/49[c]
	Hepatocellular adenoma	2/50	5/50	[illegible]/49
	Hepatocellular carcinoma or adenoma	3/50	19/50[d]	[illegible]/49[d]

[a] $B6C3F_1$ mice were fed diets containing 0 (control), 12,000 (low dose) or 25,000 ppm (high dose) of DEHA for approximately two years. The ratios of mice bearing liver tumors to the total number of mice examined microscopically are depicted.

[b] Significantly greater than controls, $p < 0.05$.

[c] Significantly greater than controls, $p < 0.005$.

[d] Significantly greater than controls, $p < 0.001$.

Table 3. Incidences of female rats with tumors of the hematopoietic system in the carcinogenicity bioassay of butyl benzyl phthlate.[a]

Hematopoietic system tumor	Incidence		
	Control	Low dose	High dose
Myelomonocytic leukemia	7/49	7/49	18/50[b]
Lymphoma	0/49	0/49	1/50
Myelomonocytic leukemia or lymphoma	7/49	7/49	19/50[b]

[a] Female Fischer 344 rats were fed diets containing 0 (controls), 6,000 (low dose) or 12,000 ppm (high dose) of BBP for approximately two years. The ratios of female rats bearing tumors of the hematopoietic system to the total number of female rats examined microscopically are depicted.

[b] Significantly greater than controls, $p < 0.05$.

mice, the data were judged to be evidence of probable carcinogenicity of DEHA in male B6C3F$_1$ mice. Carcinogenic effects of DEHA in Fischer 344 rats was not demonstrated.

Although of statistical significance, the increased incidence of myelomonocytic leukemia in female rats receiving the high dose of BBP was considered to be of equivocal biological significance due to considerable variation in the background incidence of myelomonocytic leukemia in Fischer 344 rats. Moreover, the organ distribution and cytological characteristics of the leukemia occurring in the high-dose BBP-treated female rats did not differ from that in the controls, indicating that the form of leukemia in the treated rats was the same as that occurring naturally in this strain of rat. The data, therefore, were considered to be evidence of probable carcinogenicity of BBP in female Fischer 344 rats. The study was judged to be inadequate in male rats because of chemically induced early deaths. No evidence of BBP carcinogenicity was observed in B6C3F$_1$ mice.

In summary, two of the five phthalate esters and related compounds tested by the National Toxicology Program and the National Cancer Institute for carcinogenic potential in rodent feeding studies, DEHP and DEHA, were judged to be carcinogenic. DEHP caused liver tumors in both rats and mice, while DEHA caused liver tumors in mice, only. Because of the similarities in chemical structure and the site of induced tumor formation, it can be speculated that DEHP and DEHA act through similar mechanisms. BBP was causally associated with an increased frequency of myelomonocytic leukemia in female Fischer 344 rats, but the characteristics of the induced tumor precluded an unequivocal evaluation of carcinogenic potential. The final two chemicals, phthalamide and phthalic anhydride, exhibited no evidence of carcinogenicity in these studies.

The authors acknowledge the contributions of personnel at the National Toxicology Program, Toxicology Research and Testing Program, Research Triangle Park, N.C.; the National Cancer Institute, Division of Cancer Cause and Prevention, Carcinogenesis Program, Bethesda, Md.; Frederick Cancer Research Center, Frederick, Md.; E. G. and G. Mason Research Institute, Worcester, Mass.; and Tracor Jitco Inc., Research Triangle Park, N. C., in the planning, conduct and evaluation of these studies.

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Commentary

Environmental Health Perspectives
Vol. 45, pp. 135-136, 1982

TSCA Interagency Testing Committee Actions Related to Phthalates

by Vera W. Hudson*

The Interagency Testing Committee was established by the Toxic Substances Control Act in October 1976 and has the statutory responsibility to identify and recommend to the EPA Administrator chemical substances or mixtures that should be given high priority for testing to determine the hazard to human health and to the environment. Under TSCA, testing is the responsibility of industry.

The TSCA Interagency Testing Committee had 6 months (April 1977 to October 1977) to compile the Preliminary List. This list of approximately 300 substances, mixture or categories was published in July 1977. On this list appeared the alkyl phthalates (short- and long-chain). Comments were requested on the procedures used to develop the list and on specific items on the list (*1*). At that time the TSCA Chemical Substance Inventory was not ready. Chemicals on the initial list were then screened for their potential for adverse effects in humans and in the environment. A dossier was prepared on alkyl phthalates by the suppórting contractor and information on the phthalates listed in Table 1 was presented.

After detailed review by the Committee, the category was recommended for environmental effects testing in the Initial Report dated October 12, 1977. The category was defined by the Committee as all alkyl esters of 1,2-benzenedicarboxylic acid produced in quantities of 10 million lb/yr or greater. The potential for exposure due to phthalates reaching the aquatic environment, persistence and accumulation in aquatic organisms were major causes for concern. Some phthalates are produced in volumes over 100 million lb/yr and are widely used as plasticizers in a variety of products.

Table 1. Alkyl phthalates.

Short chain	Long chain
Dibutyl phthalate	Di(2-ethylhexyl) phthalate
Diethyl phthalate	Dicyclohexyl phthalate
Dimethyl phthalate	Diisodecyl phthalate
	Diisooctyl phthalate
	Dioctyl phthalate
	Ditridecyl phthalate
	N-Octyl *n*-decyl phthalate

As a result of the second scoring exercise (1979), benzyl butyl phthalate (BBP) and butyl glycolyl butyl phthalate (BGBP) were selected for further study. Hazard Information Reviews were prepared by the support contractor. After Committee review both were recommended for testing in the Seventh Report to the EPA Administration in October 1980. Butyl glycolyl butyl phthalate was recommended because of its high exposure potential and inconclusive findings suggesting leukemia in a long-term rat study and in another study, results suggesting reproductive effects. In the case of butyl glycolyl butyl phthalate (BGBP), mutagenic and short-term genotoxic testing was recommended because of inconclusive test results. Existing data on BGBP and other phthalates indicate the need to determine the compound's potential reproductive effects. Both BBP and BGBP were recommended for environmental effects testing because little information is available on this effect. Structurally, similar phthalate esters are toxic to aquatic organisms in parts per billion ranges and have the potential to bioaccumulate.

In the third scoring exercise (1980), dimethyl phthalate and butyl 2-ethylhexyl phthalate were selected for further evaluation (*2*). Recent informa-

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Table 2. ITC recommendations on phthalates.

Category or chemical	Recommended studies	Date of recommendation
Alkyl phthalates	Environmental effects	Initial Report Oct. 12, 1977 (*3*)
Benzyl butyl phthalate	Carcinogenicity Reproductive effects Environmental effects	Seventh Report Nov. 25, 1980 (*4*)
Butyl glycolyl butyl phthalate	Mutagenicity and other short-term tests for genotoxic effects Reproductive effects Environmental effects	Seventh Report Nov. 25, 1980 (*4*)

tion generated by various groups suggests that health effects of this group may be of concern. However, the Committee has deferred any further recommendation related to health effects testing for individual phthalates or category until after this Conference. The Committee will take into account all the information presented at this meeting.

The TSCA ITC recommendations on phthalates are summarized in Table 2.

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Commentary

Environmental Health Perspectives
Vol. 45, pp. 137-139, 1982

Activities of the Environmental Protection Agency Concerning Phthalate Esters

by Steven D. Newburg-Rinn*

EPA's activities concerning phthalate esters have been in four general areas, namely: (1) their status as toxic pollutants under the Clean Water Act; (2) their status as "new chemicals" under Section 5 of TSCA; (3) the potential risk to human beings posed by DEHP; and (4) finally, the need for testing phthalates with respect to their health and environmental effects.

Clean Water Act

Pursuant to Section 304(a)(1) of the Clean Water Act, EPA is required periodically to review and publish criteria for ambient water quality. Under the authority of this section of the Act and in satisfaction of the Settlement Agreement in Natural Resources Defense Council, et al., v. Train, 8 E.R.C. 2120 (1976), modified, 12 E.R.C. 1833 (D.D.C. 1979), EPA is required to publish criteria documents for 65 pollutants which Congress, in the 1977 amendment to the Act, designated as toxic under Section 307(a)(1). Among the 65 designated compounds were the phthalate esters. In November, 1980, EPA's Office of Water Regulations and Standards made available an ambient water quality criteria document for several of the phthalate esters. These Section 304(a)(1) criteria are not rules and they have no regulatory impact. Rather, these criteria present scientific data and guidance on the environmental effects of pollutants which can be useful to derive regulatory requirements.

Under the Clean Water Act, these regulatory requirements may include the promulgation of water quality-based effluent limitations under Section 302, water quality standards under Section 303, or toxic pollutant effluent standards under Section 307. States are encouraged to incorporate criteria for toxic pollutants, based on this guidance, into their water quality standards. To date EPA has promulgated no standards for the phthalate esters.

The Water Quality Criteria Document for Phthalate Esters was prepared prior to the public release of NCI/NTP's di(2-ethylhexyl) phthalate (DEHP) bioassay results and therefore did not take into account the NCI/NTP finding that DEHP was a carcinogen in both rats and mice under the conditions of the test (*1*). The Office of Water Regulations and Standards is now in the process of updating the criteria document to reflect these recent results.

The Office of Water Regulations and Standards is also now considering a petition from Monsanto to remove butyl benzyl phthalate (BBP) from the priority pollutants list. No decision has yet been made on this petition.

Premanufacture Review Notices Under Section 5 of the Toxic Substances Control Act (TSCA)

On December 13, 1979, the EPA was given notice under Section 5 of TSCA of an intent to manufacture (PMNs) six new chemicals, described as short-chain polymers of phthalic acid and various mixed alcohols and dihydric alcohols. What I am able to say about these PMNs is somewhat limited by

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confidentiality claims. Nevertheless, the basic issues are not confidential business information and I will briefly outline the facts. No health or environmental effects data were submitted by the manufacturer with its notice of intent to manufacture. However, the similarity of the PMN substances to alkyl phthalates allowed the Agency to use data on existing alkyl phthalates to guide its preliminary assessment of the PMN substances. Also during the review period for the PMN substances the Agency received preliminary reports concerning the results of the NCI/NTP DEHP bioassay (*1*). In view of the evidence of adverse health and environmental effects of certain existing alkyl phthalates and the structural similarities of the PMN substances to those compounds, the Administrator issued an order under Section 5(e) of TSCA prohibiting the manufacture of these substances pending the submission of data by the manufacturer to clarify and define their potential hazards. Instead of going forward with the process necessary to either challenge or comply with the Section 5(e) order, the manufacturer chose instead to withdraw its request to manufacture these six substances.

DEHP Assessment Activities

Having received the preliminary indication from the NCI/NTP bioassay that DEHP was carcinogenic in rats and mice, the Agency also began a review of the potential cancer risk to humans that DEHP might pose. Questions concerning DEHP's carcinogenic potential are complex, as I am sure that the agenda of this conference makes clear. The Office of Toxic Substances has developed a preliminary risk assessment of DEHP which will be presented elsewhere in this program (*2*). I do want to note, however, that at this time questions concerning this preliminary evaluation of the potential risk to humans posed by exposure to DEHP are under review at the highest levels of EPA. Because no Agency-wide decision on DEHP's risk has yet been made, I want to emphasize the preliminary nature of this risk assessment of DEHP, and caution you against interpreting these views as EPA's position as an Agency on DEHP's risk to humans.

Phthalate Testing Needs

The Interagency Testing Committee (ITC), established under Section 4(e) of TSCA, recommended in its First Report that the alkyl phthalates be tested with respect to their environmental effects (*3*). They noted the somewhat ubiquitous nature of environmental phthalate exposure. In its Seventh Report (submitted to the Agency after the preliminary DEHP bioassay results were available), the ITC added butyl benzyl phthalate and butyl glycolyl butyl phthalate to the priority testing list under TSCA, this time recommending both health and environmental effects testing (*4*).

Talks between industry and EPA concerning phthalate testing needs began in August of 1979 with a detailed submission from Monsanto on their environmental testing program for the alkyl phthalates (*5*). Monsanto already had an environmental testing program in progress and they were interested in and willing to undertake additional voluntary testing for environmental effects.

Although the Agency was in the process of evaluating health effects data on alkyl phthalates, health effects were generally not perceived at that time as a strong concern for that class of compounds by either the Agency or industry. As these discussions continued it became apparent that a joint effort by the phthalate manufacturers would be appropriate to broaden the scope of the testing proposals. Monsanto agreed to meet with other phthalate manufacturers through the Chemical Manufacturers Association (CMA) to explore the possibility of a joint testing program. Their efforts were quite successful and a meeting was set up between EPA and industry which included representatives from the CMA and five companies manufacturing phthalates. This meeting marked the beginning of CMA's key role in industry and EPA testing discussions, which initially were focused on environmental and exposure concerns.

In early 1980, the Agency became aware of the results of the NCI/NTP bioassay (1) on DEHP, showing it to be a carcinogen in rats and mice under the conditions of the test, and (2) on butyl benzyl phthalate, where the results were equivocal under the test conditions (*6*). The DEHP results heightened the concerns of the Office of Toxic Substances, as well as those of the phthalate manufacturers, over the possible human health risks of the alkyl phthalates. A program for testing for both health and environmental effects was therefore developed by the manufacturers and users and coordinated by the CMA (forming the Phthalate Esters Program Panel). A summary presentation of this program was made to the Agency in November of 1980, followed by more detailed discussions. From the start, the CMA program was reasonably comprehensive in the environmental area. However, the health effects testing proposed at that time was limited to a small group of compounds with a focus on elucidating the mechanism of action for DEHP regarding its oncogenicity. Further meetings, then, to expand the health effects testing and also to add some additional testing to the environmental effects program were held, beginning in December of 1980, and continuing up until the present.

As a final result of all of these meetings, the Agency and the CMA appear to be close to an agreement on an industry-sponsored program of testing which matches the major testing needs of the Agency. Industry's voluntary program will answer environmental effects questions relating to toxicity to aquatic organisms, environmental fate and transport and biodegradation of all fourteen identified high production alkyl phthalates and benzyl butyl phthalate. These compounds are produced in quantities near or greater than ten million pounds per year. As will be explained elsewhere in this program (*7*), the CMA proposal also includes a screening program to examine the health effects issues of oncogenicity and mutagenicity for a somewhat smaller group of nine alkyl phthalates and benzyl butyl phthalate (*8*). Selection of these compounds was based on both production and chemical structure considerations. Positive results for a given chemical will result in additional, long-term testing and screening of more chemicals in that particular chemical's subcategory. It is proposed by CMA that cooperation of the Agency and industry extend through the testing program. When CMA's detailed proposal is received by the Agency in the near future, we will be examining it very carefully to determine whether these voluntary efforts will make necessary a regulation requiring the health and environmental effects testing of the phthalate esters.

A few notes about the CMA health testing program are in order. It may appear on first impression that EPA's needs for testing differ from CMA's. I do not think this is so. Both industry and EPA need to know more about phthalate toxicity, and government has no investment in phthalates being found to be either human health or environmental risks. While the CMA program is clearly experimental, it clearly could establish the toxicity of phthalates in addition to DEHP. I believe that our knowledge of phthalate toxicity will appreciably advance if CMA goes forward with this program. (Since this conference, the EPA has accepted, as of October 30, 1981, a CMA proposal to do health and environmental effects testing of the alkyl phthalates on a voluntary basis.)

The author gratefully appreciates the skilled assistance of Mr. John Schaeffer, Ms. Debbie Michelson and Ms. Dorothy Leysath.

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Commentary

Environmental Health Perspectives
Vol. 45, p. 141, 1982

National Institute for Occupational Safety and Health: Areas of Concern

by P. Jackson Schad*

The National Institute for Occupational Safety and Health (NIOSH) is evaluating potential human health risks that might result from workplace exposures to phthalate esters, including the following workplace applications of di(2-ethylhexyl) phthalate (DEHP): (1) respirator quantitative fit testing (QNFT); (2) plasticizers; (3) air-filtration system leakage tests.

QNFT is routinely performed to determine respirator facial fit. A polydisperse DEHP aerosol is nebulized in QNFT and incorporated as a test atmosphere to quantify respirator faceseal leakage. However, emerging evidence of phthalate toxicity prompted NIOSH to question the current practice of using a DEHP aerosol in QNFT and whether alternate test agents may be substituted for DEHP in the procedure. NIOSH has tested several alternate agents to evaluate their aerosol-generating properties in existing QNFT equipment made for DEHP. Our results have indicated that at least three substances, including aerosols generated with refined corn oil, di(2-ethylhexyl) sebacate (DEHS), and dimethicone, qualify to replace DEHP. Furthermore, all the data examined have indicated that refined corn oil stands apart from the other proposed agents and currently represents the best option for DEHP replacement.

A review of the toxicities and aerosol properties of both DEHP and candidate substitutes will be described in a forthcoming NIOSH peer reviewed report projected for publication in 1982. The report will recommend refined corn oil for interim DEHP substitution in the QNFT procedure. In conjunction with this report, NIOSH is conducting acute and subchronic inhalation toxicity testing of refined corn oil and DEHS in rodents at the Johns Hopkins University. Animals will be exposed to aerosols of either substance nebulized by the same equipment used in QNFT. Doses for the subchronic exposure will be determined in the acute phase of the experimental protocol. Testing began in October 1981 and will take 18 months for completion.

Another occupational application of phthalates under evaluation is the extensive use of DEHP as a plasticizer. Our review has concluded that the plastics industry may not be characterized adequately for DEHP exposures, although the exposure potential may be low due to a high degree of mechanization in this industry. NIOSH field investigators are currently reviewing potential areas where DEHP plasticizer exposures might occur. If a suitable cohort is identified, NIOSH will consider a joint epidemiologic study with the U.S. Environmental Protection Agency. Results of our preliminary survey are anticipated by June 1982.

Occupational exposure to thermally generated aerosols has also been considered in the selection of a cohort for the proposed epidemiologic study. NIOSH is presently evaluating worker exposures to a monodisperse, thermally generated DEHP aerosol, widely used in a test procedure to detect leaks in air-filtration systems. However, breakdown products from the thermally treated DEHP could contribute confounding factors to the assessment of human health effects. Accordingly, NIOSH believes that a better candidate for study would be a filter test utilizing DEHP nebulized at room temperature rather than a thermally generated aerosol.

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Commentary

Environmental Health Perspectives
Vol. 45, pp. 143-144, 1982

Safety of DEHP: Role of the Bureau of Biologics

by Joseph C. Fratantoni*

The position of the Bureau of Biologics (BoB) relative to the issue at hand is unique on several counts. First, Bureau of Biologics is formally interested in the phthalate issue only in connection with containers for blood and blood products. The authority to regulate the nature of such containers derives from specific regulations. For example, regarding plasma, 21 CFR, Section 648.68(b) states: "Final containers and their components shall not interact with the plasma contents under conditions of storage and use so as to alter the safety, quality, purity or potency of the plasma."

Second, the subjects involved are exposed to the highest doses of DEHP, and it is delivered via the intravenous route. Most of these subjects are patients undergoing treatment, although the increased interest in pheresis procedures will result in similar exposure for more and more normal donors.

Finally, BoB has considered the DEHP in vinyl plastics to be a problem for a number of years. That is, we have been aware that DEHP, a biologically active material, was leached by blood and plasma in considerable quantities from the PVC bags. We were also aware that the available data on toxicity did not support a regulatory demand for immediate change. Members of the blood banking community were similarly concerned, and this concern was shared by the manufacturers of blood bags, as indicated by the considerable research expenditures dedicated to the quest for a substitute plastic.

At BoB the concerns which derived from the presence of DEHP were balanced by an appreciation of the need for a flexible container. The entire science of blood component preparation in a sealed system is dependent upon a flexible container. The Bureau of Biologics would therefore consider that reversion to a system based on glass bottles is not a viable alternative.

As far as actions that have been taken and will be taken, I would like to refer to the interesting dialogue at this conference between Drs. Peck and Kevy regarding the ability to show that any of the deposition of DEHP in mammalian organs led to demonstrable toxicity. I believe that both Drs. Peck and Kevy would agree that another question is appropriate and that is, rather than asking, "Is it toxic?" the question might be, "Is it possible to demonstrate toxicity in the clinical situation?" I further believe they would both agree that, given the difficulties of clinical investigation, it is not possible to answer that question. So, for the immediate future, the BoB will not await a definitive answer to the toxicity question, but will address other approaches to the problem.

We are aware that there is some concern about the rapidity with which submissions are reviewed and approved. Accordingly, we have already undergone some discussion, namely, blood and blood product containers, both dry and with anticoagulant. Specifically, after a period of about two years of investigating and holding conferences on the subject of testing blood platelets, we have simplified the procedures which were being demanded of the manufacturer for obtaining approval of a container used for storage of platelets. It had been the opinion of many in the community that this particular type of testing was the most onerous. We have developed a new set of guidelines which are available to any interested person and can be obtained by writing to the Director, Bureau of Biologics.

We will encourage accumulation of data on the content of DEHP in various blood products and hope, some time in the foreseeable future, to be able to encourage the development of guidelines for blood banking practice. Since we need a flexible bag, and the currently available systems have large amounts of leachable plasticizers, we would hope to limit exposure by some simple expedient such as using the shortest storage time possible for those components where this is practical. Knowledge of the DEHP content of various components, correlated with the conditions for their preparation and storage, should suggest a variety of maneuvers which would decrease patient exposure to infused DEHP.

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We certainly agree that more fundamental data regarding carcinogenicity of DEHP are needed. Given the problems with the assay system, the variations in the literature stated, and species differences that we have heard about, we believe that any quantitative assessments of risk are premature at this time. We have been opposed to disseminating such details to patient such as hemophiliacs and leukemics, whom w feel are sufficiently burdened, and need not be oncerned with questionable numbers at this time

Finally, regarding the question that w s asked in the previous session about a timetable f action. In addition to what has been said, I can onl state that the Bureau of Biologics will take fur er action when we feel that there is a consensus ithin the scientific community that it is time to d o. At this time, we do not feel that there is such a nsensus.

pages tightly bound

Commentary

Environmental Health Perspectives
Vol. 45, pp. 145-147, 1982

Interagency Regulatory Liaison Group Role in Phthalates

by Phillip A. Johns*

Introduction

In its attempt to regulate interstate commerce of chemicals within the United States, Congress created five regulatory agencies with the burden of overseeing various aspects of that trade. The Environmental Protection Agency (EPA), the Consumer Product Safety Commission (CPSC), the United States Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS) the U.S. Department of Labor and its Occupational Safety and Health Administration (OSHA) and the Food and Drug Administration (FDA) regulate the use of chemicals in interstate commerce. Because each of these agencies arose through different enabling legislation and are concerned with a different use of chemicals, it is likely that the same chemical would be independently regulated by two or more of those agencies. In addition, because chemicals and their manufacture and use, in general, are a concern of each of those agencies, policies and requirements for safe use of those chemicals could be different for each agency. For the above reasons, in October 1977, at the urging of the executive branch, each of the agency heads of EPA, CPSC, FDA and OSHA agreed to recommend those specific areas in which interagency cooperation would be beneficial to all agencies. Some areas of concern were finally agreed upon by the agency heads.

In the area of toxicity testing guidelines, it was important for each agency to require similar standards to prevent duplicative testing by industry. Cooperation could result in a development of standardized testing guidelines which, if utilized in a toxicity test, would be acceptable for safety standards among the agencies. Joint research planning was another area where common interests could be identified. Each agency could avoid unnecessary duplication in spending for research already being performed by another agency. The third area was information exchange. The objective was to improve the quantity and quality of information available to each agency. Risk assessment was a fourth area identified since it is essential that health and safety decisions among agencies be made in a uniform manner. It is also important that risk assessment made by uniform decision making, identify potential problem areas. Fifth, it was also believed that an inspector from a single agency could observe the same territory as many inspectors from different agencies had previously. This attempt to coordinate inspection efforts was believed to increase the efficiency and reduce the burden of inspection on industry and government. Sixth, the agency heads wanted to determine if there were sufficient resources that could be pooled to develop a uniform federal government agency response to problems of common concern to two or more agencies. Communication and education were considered important because it was felt that information transmitted to the public on toxic substances should be consistent, accurate, and educational. The development of a uniform policy for review of epidemiology data was the eighth area where the agency heads felt that there was common interest.

In October 1977, the agency heads formally announced the creation of the Interagency Regulatory Liaison Group (IRLG). The stated goal of that organization was "to increase the public health and protect the environment by sharing information, avoiding duplication of effort and developing consistent regulatory policy while reducing the burden on those regulated and the agencies themselves (*1*).

It was decided to concentrate on research for regulation of hazardous substances on a nationwide basis. Some operating concepts are to avoid creating another level of bureaucracy, to focus on action, not studies. Coordination is the rule, not the exception. It was deemed important to be certain

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Table 1. Chronology of development of Interagency Regulatory Liaison Group (IRLG) comprising Consumer Product Safety Commission, Environmental Protection Agency, Food And Drug Administration, HEW, Food Safety And Quality Service, USDA, Occupational Safety And Health Administration, DOL.

Date	Step
August 2, 1977	Announced intention to form IRLG
September 26, 1977	Signed agreement
October 11, 1977	Published agreement in *Federal Register*
February 17, 1978	Published work plans in *Federal Register*
March2-6, 1978	Work groups held public meetings
January, 1979	FSQS joined IRLG
May 22-23, 1979	Agency heads held public meetings

that arrangements make good sense and to emphasize quality regional coordination.

In September 1977, an interagency agreement was signed by each agency head to commence this newly created "Confederation of Agencies." Draft work/action plans were prepared and published in the *Federal Register* in February 1978 (*2*). Finally in January 1979, the Department of Agriculture/Food Safety and Inspection Service, as it is now known, joined the IRLG (Table 1).

Organization of IRLG

IRLG as a group of agencies has only one fulltime employee. This is to reinforce the idea that agency employees are also members of the IRLG. Each member agency designates part of its operating budget for the funding of IRLG programs.

The IRLG is directed by the five agency heads. Day-to-day activities of the agencies are overseen by two surrogates from each agency. The substructure below the surrogates consists of regional staff, task groups, work groups, senior headquarters staff and operating staff, but there is no set organizational hierarchy as to how these groups interact. The organization is flexible to allow for unexpected emergencies where one group will be required to interact with another.

Regulatory development is one of the groups within working groups. It is a work group consisting of 20 subwork groups which each are concerned with a chemical of concern to at least two of the member agencies (Table 2). Twice a year each work group publishes a report on their activities in a free booklet called *The Regulatory Reporter*. Copies of the *Regulatory Reporter* are available from EPA; Office of Pesticides and Toxic Substances; Industry Assistance Office; 401 M Street S.W.; Washington, D.C. 20460, (800) 424-9065 and in Washington, D.C. 554-1404.

Table 2. Work groups and counterpart groups.

Work Groups	Counterpart Groups	Regions
Epidemiology	Budget	I: Boston
Information Exchange	Compliance and Enforcement	II: New York
Regulatory Development	Congressional Affairs	III: Philadelphia
Testing Standards And Guidelines	Contracts	IV: Atlanta
Education Task Force	Economists	V: Chicago
	Freedom Of Information	VI: Dallas
	General Counsels	VII: Kansas City
	Personnel	VIII: Denver
	Planning and Evaluation	IX: San Francisco
	Public Information	X: Seattle
	Regional Coordinators	
	Research Planning	

The Phthalate Workgroup on Plasticizers

Soon after the results of National Cancer Institute Carcinogenic Bioassay on di(2-ethylhexyl) phthalate (DEHP), di(2-ethylhexyl) adipate (DEHA) and butyl benzyl phthalate (BBP) became available in draft form, it became apparent that each of the member agencies of IRLG would be concerned about the results and possibly interested in future reregulation of those compounds based on the NCI data.

IRLG immediately responded by forming a new work group on plasticizers. Each Member agency appointed a representative (it should be apparent that if a particular agency had no interest in the regulation of a compound, it would not appoint a representative). Because of the diverse interests in plasticizers, the work group has had representatives of a number of non-IRLG agencies including the Department of Defense (DOD), the National Toxicology Program (NTP), The National Institute of Occupational Health and Safety (NIOSH), and the Nuclear Regulatory Commission (NRC), attend its meetings. The work group agreed that DEHP, DEHA and BBP were of primary concern; however, any possible alternative for those compounds would also fall within the review by the work group. It was also of general consensus that all toxicology and exposure data available within an individual agency would be exchanged so that the same data base would be considered by all involved.

It was obvious that the data base for toxicity studies on phthalates was large. However, the only long-term studies on phthalates other than the recent NCI studies were completed in the middle 1950s. Although negative in terms of carcinogenicity, those studies were not conducted under present day testing standards. It was a concern of the IRLG scientist representing each agency that advice from outside the U.S. government on the generally accepted interpretation of those results be sought out. As a result, the work group agreed that a conference consisting of recognized experts in the field of phthalates and carcinogenesis could provide the work group and member agencies enough background on manufacturing, exposure, toxicology and testing rationale data so that regulatory decisions on the matter could be made by each agency.

Agreements were made between the NTP and IRLG who sponsored this conference on phthalates. The work group felt that a conference held prior to commencement of any formal regulatory action would allow participants to freely air their views without a cloud of pending regulatory activity which might restrict the free flow of ideas.

The information gained from the conference is presently being considered by the agencies and work group. The information was valuable and helped the work group understand many of the issues involved with the problem.

The workgroup is now continuing its efforts by working with the NTP and the Chemical Manufacturers Association to develop a clearinghouse for ongoing toxicological studies being conducted throughout the world. This program is presently being initiated and it is hopeful that it can prevent duplication of experimentation by scientists throughout the world and continue the open dialogue for discussion of the interpretation of these new results.

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Environmental Health Perspectives
Vol. 45, pp. 149-153, 1982

Discussion and Summary Remarks*

Bioassays for Carcinogenicity

Much of this conference has concerned itself with a single chemical, di(2-ethylhexyl) phthalate (DEHP). The National Cancer Institute (NCI)/National Toxicology Program (NTP) Bioassay Program, however, has tested a total of five phthalate esters or related compounds for carcinogenic potential. These are reviewed briefly below.

Phthalamide

Phthalamide (NCI TR-161) was found to be of low acute toxicity, allowing it to be tested in mice and rats at doses of up to 3% (by weight) in the diet. Toxic lesions were produced in the livers and urinary tracts of both species, but there was no evidence that phthalamide was carcinogenic. Considerable numbers of animals were diagnosed as having hyperplasia of the urinary bladder, and two of the high-dose female rats had transitional cell carcinomas of the urinary bladder. The report mentions excess calculus formation in the urine, but it is not clear whether that represents precipitated test substance, mineral deposit, or inflammatory exudate that may have been inspissated. Hyperplasia of the bladder mucosa may have been related to the presence of foreign material. On the other hand, it may also have represented focal hyperplasia with dysplasia related to compound administration. A possible limitation in this report is that the control groups are rather small with only 20 animals per group. The test would have been more sensitive with larger control groups. Testicular atrophy cannot be adequately assessed in chronic studies with the Fischer 344 rat because this strain develops a virtually 100% incidence of interstitial cell tumors in the second year of life, accompanied by germinal cell atrophy. Testicular effects could be addressed with early sacrifices or, better yet, with a different strain of rats.

Phthalic Anhydride

Phthalic anhydride (NCI TR-159) was administered in the diet at doses of up to 5%, and even this level did not appear to be a maximum tolerated dose in the female rats. The test diets were formulated every one or one-and-a-half weeks, even though as much as 25% of the test compound was shown to decompose upon storage for 10 days. The exact doses administered, therefore, are unknown. The only possible evidence for carcinogenicity from phthalic anhydride in this bioassay was the occurrence of excess leukemia in the female rats in the low-dose group. This was interpreted by the authors as not being significant because an excess of leukemia was not found in the corresponding high-dose group. It should be noted, however, that of all the possible cancer end points in the Fischer 344 rat, leukemia is the most variable both in incidence and in time to onset. Additional experimentation with larger groups of animals may be required to evaluate the possible leukemogenicity of phthalic anhydride.

Di(2-ethylhexyl) Phthalate

The report on di(2-ethylhexyl) phthalate (DEHP) has already been reviewed in this conference at some length. An unanswered question in this bioassay is whether palatability or toxicity was the cause of decreased weight gains. Interstitial cell tumors of the testis in male rats were decreased in animals administered the higher dose of DEHP. In contrast to the control group, which had 96% testicular tumors, those administered the high dose of DEHP had only 23% testicular tumors. The rats administered the high dose weighed less and may have had a lower tumor background incidence related to body weight. It is more likely, however, that DEHP had an inhibitory effect on testicular tumor formation, and this finding deserves further study. The weight of the evidence for the carcinogenicity of DEHP is very strong. The excess of liver tumors in both sexes of both species in a dose-related manner, with supporting data from *in vitro* transformation experiments, is conclusive.

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Di(2-ethylhexyl) Adipate

The bioassay report on di(2-ethylhexyl) adipate (DEHA) indicated that the test chemical produced liver tumors in female mice and possibly in male mice. Not emphasized in the report were early deaths in male mice administered the low dose of the test substance. In the first year of the experiment 22% of this group of animals died from unexplained causes, thus limiting the number of animals at risk to late developing tumors. Liver tumors were found as early as 37 weeks after the beginning of test compound administration, however, suggesting that further evaluation, including age adjusted analyses, may make it possible to reach a more definitive conclusion.

Butyl Benzyl Phthalate

The fifth bioassay conducted by the NCI/NTP was that of butyl benzyl phthalate. The authors of this report concluded that butyl benzyl phthalate may have produced leukemias in female Fischer 344 rats, as they occurred more frequently in the treated animals than in controls. The tumors were of the myelomonocytic type commonly found in the Fischer rat, however, and the background incidence of this tumor type in Fischer rats has been found to be variable. Another experiment, with larger groups of animals, may indicate whether or not butyl benzyl phthalate is capable of producing leukemia in the female Fischer 344 rat. Both testicular and thymic atrophy were reported in butyl benzyl phthalate-treated rats. Testicular atrophy secondary to phthalate ester administration has previously been noted, but the finding of thymic atrophy indicates that immunotoxicological studies should also be considered. The NTP has indicated that the apparent hemorrhage-induced deaths produced in male rats by butyl benzyl phthalate will be explored in another chronic study, with attention given to both disorders of the clotting mechanism of the blood vessels as well as to carcinogenic potential.

Critique

The NCI/NTP Bioassay Program, including the testing of phthalate esters and related compounds, has proved invaluable in identifying chemicals with carcinogenic potential. Review of the technical reports, however (many of which were, admittedly, only in draft form), indicates that improvement could be made in their contents. More specifically, tabular data need to be rigorously checked for accuracy, experimental findings should be described more completely and the discussion sections ought to be expanded greatly to provide readers with a wider perspective on the chemical under test and explanations for the effects produced.

The descriptive studies of the NCI/NTP have identified a potential problem, the carcinogenicity of phthalate esters. Chronic bioassays, however, do not elucidate the molecular mechanisms of toxicity that are needed to provide credible bases for estimating human risk. Future studies, therefore, should be directed towards the mechanistic aspects of the biological effects of DEHP. A determination of whether DEHP exerts any genotoxic effect or directly damages DNA is a logical starting point. This appears to be an important issue in relation to contradictory results which were obtained in short-term tests in bacteria. It would be very useful to know whether these agents bind covalently to DNA (and, if so, what kind of binding occurs), and whether the radioactivity incorporated into DNA depends upon metabolic activation. This is important not only in the understanding of how these agents may act, but also in interpreting results from the various short-term tests. The simple addition of various types of short-term tests to a study does not necessarily improve the quality of the data that is obtained, nor does it help greatly in interpreting the possible risk of the agent, especially if the series of short-term tests is based upon the same end point. Thus, there is a risk that by adding a lot of information on short-term tests without having parallel studies on the mechanism of action, more confusion than help will be created. A balance between the two approaches appears to be essential.

In vitro cell transformation, on the other hand, may or may not be the result of a DNA-damaging event. It would be interesting to determine whether cell transformation in culture is paralleled by increased peroxisome proliferation with this type of agent or with the hypolipidemic drugs. Many carcinogens induce DNA damage and this damage has been causally associated with carcinogenesis. At the same time, however, it should be noted that some carcinogens do not necessarily act through this mechanism. For example, diethylstilbestrol (DES) is carcinogenic in humans and causes cell transformation, but there is only very weak evidence that it binds to DNA, and it is not mutagenic (*1*, *2*). Another example is TCDD, which has been examined for its capacity to bind to DNA by Poland and Glover (*3*), who showed that none existed. Yet this chemical was a hepatocarcinogen for rats. From the public health point of view, this type of agent which induces tumors apparently through a mechanism not involving DNA damage should not be considered less dangerous than DNA damaging agents.

Despite these potential problems in the interpretation of mechanistic studies, it is generally felt that such information can provide a more technically sound and scientifically relevant basis for risk assessment than the purely descriptive studies. The bioassay studies, therefore, can be viewed as providing clues to the understanding of the toxic potential of chronic exposure to phthalate esters.

The technical difficulties and high costs involved in mechanistic studies preclude the performance and duplication of each and every possible experiment. It is apparent from this conference, however, that several investigative groups throughout the world are currently engaged in the safety evaluation of phthalate esters. Every attempt should be make to keep all of these groups in close communication regarding the outcomes of the experimental work.*

Short-Term Tests and Their Role in Assessing the Toxic Potential of Phthalate Esters

The Chemical Manufacturers Association (CMA) has proposed a battery of short-term tests to be used in determining which additional phthalate esters ought to be tested for carcinogenicity, and the NTP has outlined a program in progress concerning short-term testing of these compounds. If short-term tests are to be used as predictors of carcinogenicity, then those to be considered should be point mutations in mammalian cells, point mutations in microbial cells, and *in vitro* transformation. This was the grouping of tests suggested by the Interagency Testing Committee some two years ago. Unscheduled DNA synthesis in mammalian cells can be used as an augmenting or supporting assay. However, results from this test system are dependent upon biological effects such as mutation or *in vivo* transformation for interpretation. If short-term tests are to be used to detect mutagenic potential leading to an evaluation of genetic risk, then chromosomal-level assays, such as *in vivo* and *in vitro* cytogenetics analysis, would have to be included.

Positive results in the first grouping of assays would suggest a need to assess carcinogenicity in rodents, while positive results in the second grouping would suggest a need to measure germ cell effects in animals. The major application of short-term tests identified by this conference is prediction of carcinogenic potential. At the present time, published results of short-term tests do not generally indicate that DEHP is genotoxic. However, test results on the monoester and the ethylhexyl alcohol are suggestive of such, though not confirmed, and the suggestion is that these components may be mutagenic. Reproducible results in different laboratories are needed to confirm these initial findings. It is essential that this scientific process be satisfied before labels of genotoxicity or lack thereof are applied.

Attention should be paid in the short-term tests to the metabolic activation systems used. For almost all classes of chemicals requiring metabolic activation, this is probably the most critical element in this type of assay. There are various types of metabolic activation systems (e.g., freshly isolated liver cells) which are used in addition to an S9 fraction. The metabolism of DEHP in most short-term test systems is unknown. At a minimum, therefore, the diester, the monoester and the alcohol—i.e., DEHP, mono(2-ethylhexyl) phthalate and 2-ethylhexanol—should be subjected to the short-term tests. The activities of other, lesser metabolites existing in higher oxidized states may have to be evaluated on a case-by-case basis.

By using *in vivo* systems, where possible, the entire spectrum of metabolites can be assessed. This brings to consideration other models for evaluating the phthalates. One assay which permits the metabolic activity of the whole animal to be expressed is the testing of urine from chemically treated animals on microbial or mammalian indicator cells. If positive, this model, coupled with appropriate analytical techniques, could identify the active metabolites. Another whole animal model which could be considered is *in vivo* cytogenetics. It may be argued that this assay is insensitive. However, it may give a truer reflection of any perceived hazard at a reasonable dose level. Further, and just as important, there appears to be a strong correlation between chemicals which are positive in mammalian germ cells—in other words, producing chromosomal level effects—and those which are also positive in *in vivo* cytogenetic assays (e.g., bone marrow cells using metaphase analysis). This is an important aspect of genetic toxicology testing which has not been fully appreciated to date. It is suggested, therefore, that compounds be tested in this model as a means of selection for dominant lethal or heritable translocation assays. Additional consideration should be given to *in vivo*/*in vitro* testing. Methods are available to expose liver cells *in vivo*, remove them and measure unscheduled DNA synthesis *in vitro*. This would focus, *in vivo*, on the liver, the organ of most concern in the carcinogenesis bioassay of DEHP.

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*In response to this need, the National Toxicology Program (NTP) and the United States Environmental Protection Agency (EPA) have organized a clearinghouse for collection and dissemination of information on phthalates. Contact: Ms. Joan Chase, International Clearinghouse on Phthalate Esters, National Toxicology Program, Landow Building, NIH, Bethesda, Md. 20205.

Finally, a note should be made on the data indicating evidence of dominant lethal effects of phthalate esters in rodents. Evaluation of these experiments are complicated by the use of extremely large, toxic doses and inadequate numbers of females and treated male animals. Many of the phthalate esters are known to damage the seminiferous tubular epithelium of the testes. Moreover, the end point in this test—fetal wastage—could result from a direct toxic, nongenetic effect on the germinal cells. The dominant lethal studies bear repeating, therefore, with the inclusion of doses that are not toxic to the testis.

Review of CMA and NTP Testing Programs

The testing program of the CMA (4) is designed to indicate whether additional phthalate esters need to undergo carcinogenicity testing in animals, while that of the NTP (5) is directed more to determining the mechanism of DEHP carcinogenicity and to evaluating the other toxic effects of phthalate esters. The complementary nature of these two programs is purposeful, rather than coincidental, reflecting a determined effort on the part of both government and industry to avoid costly duplication of effort.

Some of the less obvious points of these programs should be noted. First, if the carcinogenic potential of phthalate esters is being questioned on the basis of the DEHP chronic bioassay results, then the short-term tests utilized to prioritize such agents for carcinogenicity testing ought to be those in which DEHP is active. To date there is no concensus as to whether or not DEHP is active (positive) in short-term tests. The CMA and NTP programs wisely propose, therefore, that any battery of short-term tests used to identify phthalate esters for testing in a bioassay include tests in which DEHP is active.

Secondly, chemicals can cause cancer via nongenetic mechanisms, a likely one for DEHP (based on biological effects) is promotion. The NTP program, therefore, includes the testing of DEHP for liver tumor promotional activity. It must be recognized, however, that there is no single, standard test for liver tumor promoters nor is there any definitive indication of the mechanism of tumor promotion in the experimental systems currently available. The results of the DEHP promoter studies, therefore, no matter how well conducted, are likely to be confusing and controversial. Nonetheless, their performance may suggest a mechanism of DEHP carcinogenicity.

In addition to the CMA and NTP programs, two groups have indicated that they are currently evaluating the abilities of DEHP, mono(2-ethylhexyl) phthalate and 2-ethylhexanol to bind to liver DNA. These studies are most important not only because they may suggest a mechanism of DEHP carcinogenicity, but because they could indicate which metabolites of DEHP are causative of cancer and whether or not species differences in the metabolism of mono(2-ethylhexyl) phthalate preclude the use of rodents as valid models of human response to DEHP.

Finally, mention has been made of possible epidemiological studies of phthalate ester toxicity. While recognizing that negative epidemiological studies are often inconclusive, it is still felt that an attempt should be made to identify persons with high exposures to phthalate esters and to determine whether their health status has been compromised. Medical patients exposed to DEHP via repeated transfusions or hemodialysis are probably not an acceptable group because of numerous confounding health problems. Both cancer and infertility should be considered in any epidemiological evaluation.

Summary Remarks

It is clear from the data presented at this conference and the resulting discussion that the evaluation of chemical safety is a very complex issue. In addition to the science involved, regulatory decisions must take into account economic impacts, technical capabilities and comparative toxicities of substitutes when the safety of a chemical or a process is questioned. This conference may not simplify the decision-making burden in the case of plasticizers, but public presentation and discussion of the relevant information at least assures that the scientific aspects of the problem have been identified.

The carcinogenesis bioassay of DEHP conducted by the NCI/NTP has been discussed previously in a public forum and was reviewed and approved by a peer review group of independent scientists.* Clearly, DEHP is carcinogenic to rodents. It is recognized, however, that the evaluation of the risk to man from exposure to phthalate esters involves more than just a descriptive bioassay. Hence, the need for programs concerning mechanisms of action, pharmacokinetics, interspecies comparisons and epidemiology as discussed at this conference. These efforts are justified, particularly in the case of a chemical which is so widely used and for which there would appear to be no obvious safe substitute.

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*Meeting of the National Toxicology Program Board of Scientific Counselors' Technical Report Subcommittee and Panel of Experts, Conference Room 6, Building 31C, National Institutes of Health, Bethesda, Md., October 15, 1980.

The limited resources available for chemical safety testing necessitate a mechanism for prioritizing phthalate esters for chronic toxicity studies. Short-term tests may be helpful in this function, but their apparent inability to clearly identify DEHP as a potential carcinogen, with the exception of the recent Japanese studies, is troublesome. It may become necessary to use a different strategy to determine which phthalate esters are in need of assay for carcinogenic potential. The point has been raised at this conference of the need to determine whether DEHP caused tumors in rats and mice by a genetic or a nongenetic mechanism. This information would be helpful in understanding the toxicology of DEHP and in interpreting short-term test results, but the relative safeties of nongenotoxic carcinogens (such as promoters) in comparison to genotoxic carcinogens is not well understood. Simple labeling of chemicals as genotoxic or nongenetic carcinogens, therefore, is not sufficient for safety evaluation.

The emphasis at this conference has clearly been on the carcinogenic potential of phthalate esters. Other toxic effects, such as teratogenicity and infertility, however, should not be ignored in assessing the risk of human exposures to plasticizers. Groups such as the NTP have recognized the importance of nontumor toxic effects and increasingly are incorporating more diverse experimental protocols into their toxicological analyses. Too often the lack of studies designed to evaluate toxicity have been misinterpreted as a demonstrated lack of toxicity and we must be careful not to repeat such errors, particularly when comparing potential substitutes for phthalate esters.

It is interesting to note that in 1972 the National Institute of Environmental Health Sciences sponsored a Conference on Phthalic Acid Esters (*6*). Although carcinogenic effects were not a major issue at that time, it was felt nevertheless that the high volume of production of these chemicals and the large number of people exposed to them necessitated a clear understanding of their toxic potentials. We still find ourselves in that situation, needing more information, but with a better idea of what we are seeking. Additionally, the spirit of cooperation shown at this conference between government, industrial and independent scientists suggests that we will reach that goal with all due speed.

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Contributed Articles

Environmental Health Perspectives
Vol. 45, pp. 157-164, 1982

Lung Cancer Mortality and Residential Proximity to Industry

by Marise S. Gottlieb,* Charles L. Shear† and Daniel B. Seale†

A potential causal relationship has been suggested by other studies between air pollution and lung cancer. To attempt to define the risk of lung cancer associated with residential proximity to industry by type in Louisiana, lung cancer deaths occurring between 1960 and 1975 in residents of 20 parishes were compared to controls matched on age, sex, year of death and parish of residence. The comparisons were limited to cases (N = 1418) and controls (N = 1429) with known length of exposure to and residing within 0.99 mile (exposed and 1.0 to 3.0 miles (unexposed) radius of an industry type. Of the 13 industry types evaluated, the petroleum and chemical industries showed the highest consistent elevations in risk associated with closeness of residence to industry, whereas possible risks shown for food, grain, canning, and paper industries are less defined.

For the petroleum industry, the risk was demonstrated in the group with 10 or more years of residential exposure to the industry in question. For the chemical industry, the residential risk was found in people employed in low risk occupations, who were exposed to large individual industries and was independent of length of exposure as determined for less or more than ten years, ($\hat{RR}$ = 4.5). The results suggest that residential proximity to petrochemical industries may make a contribution to the lung cancer mortality in Louisiana.

Introduction

Lung cancer mortality and incidence in certain southern parishes of Louisiana are among the highest in the U.S. (*1-3*). There are certain unique industrial characteristics in Louisiana. Numerous petroleum refineries are situated along the Gulf Coast and in Louisiana. The petrochemical industry along the Mississippi River and related areas is about as concentrated as in any other region in the United States.

A potential causal relationship has been suggested by other studies between urban air pollution and lung cancer. A number of investigators have shown an urban-rural gradient in lung cancer mortality (*4, 5*). However, access to and availability of medical care, migration due to illness, cigarette smoking and occupation are factors associated with urban-rural residence which may distort the ability to attribute such a gradient to air pollution. Even with the present access to medical care for residents of rural areas, this gradient persists (*5*). After controlling for cigarette smoking patterns, a number of studies in different populations have shown the gradient still to be present (*6-12*). Due to the limited employment of the population in occupations identified as contributing to the risk of lung cancer, occupational hazards could not completely account for the urban-rural gradient (*5*).

Correlation analyses have shown a relationship between lung cancer mortality, benzo[a]pyrene measurements, solid-fuel consumption, and total settled dust (*4, 13*). Commoner has demonstrated that certain samples of urban air in Chicago are mutagenic (*14*). Extrapolation of results from occupational exposure to polycyclic hydrocarbons, and specifically benzo[a]pyrene have led investigators to the conclusion that lung cancer mortality may be substantially increased by exposure to air pollution (*15, 16*). Lung cancer has been shown to be associated with the percentage of the population of a county employed in the petroleum, chemical, paper,

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and transportation industries (*17*). Lloyd's report of excess lung cancer mortality in a residential community downwind from a steel foundry further supports the potential effect of air pollution (*18*).

The present report details a mortality study using a case-control methodology in which the length of exposure is estimated and distances from exposure sources are calculated while controlling for occupational exposure and some demographic variables, which strongly suggests a lung cancer risk attributable to residential proximity to industry. Because of the short length of survival from the date of diagnosis of lung cancer, mortality data can be used to approximate incidence data, and the length of residence prior to death can be used to approximate exposure to effluents before onset of disease.

Materials and Methods

Study Population

Twenty parishes (counties) in Louisiana were selected for study based on 1% or more of the population being employed in the paper, chemical and petroleum industries, and/or high rates of mortality from lung cancer (1950-1969) (*19*).

Cases were primary lung cancer deaths identified from death certificates, (ICD 162.1, 163. 7th revision; 162.1, 8th revision) occurring between 1960 and 1975 with a verified minimum length of residence in the parish of residence at death (*20*). Controls were drawn from a sampling frame which excluded deaths due to neoplasms, and were matched on parish of residence, age (± 2 years), race, sex, and year of death.

Industry of Occupation

Usual industry of occupation and occupation were abstracted from death certificates and classified according to the definitions of the Census Bureau Classification, (*21*). For this report, industry of occupation was dichotomized into high and low risk (*19*). High risk industries of occupation for lung cancer included fishing, construction (including painting), metal equipment and transportation equipment manufacturing (including shipbuilding), petroleum refining, chemical, other manufacturing (U.S. Census Bureau Codes 119-138, 248-259, 299-319, 337-398) (including electrical equipment) and transportation.

Manufacturing Industries

Manufacturing industries in operation between 1942 and 1965 were identified using the Louisiana Directory of Manufacturers (*22*). All the industries reported in the directories published between 1942 and 1975 were compiled in order to overcome the variability in reporting to individual editions. The type of industry, opening and closing dates, addresses, and average number of employees (size) were obtained. These industries were mapped on parish maps to the nearest 0.1 mile, and the location was transformed to a coordinate system.

Distance and Exposure

Place of usual residence was abstracted from the death certificate. Addresses of cases and controls were transformed by the same method as that used for industries. Information on length at residence was obtained by field workers surveying public utility records and town directories. The distances of cases and controls from an industry were calculated using the simple Pythagorean rule.

A subset of cases and controls was further defined according to known residential exposure to industrial effluents; that is, those with concurrent known dates of residence and dates of operation of the industry. Years of known exposure were calculated according to the years of overlap. This report is limited to cases and controls with known minimum years of exposure to specific types of industries prior to death. Exposure variables were considered separately rather than in combination as a single dose-response variable. Distance from industry was chosen as the primary response variable as it was the most accurately measured. Years exposure are minimum known years at residence of death during which the industry was in operation. Industry size (assumed to be proportional to effluents released) was estimated from the number of employees recorded as employed by the company.

Case-Control Comparison

In comparing case-control distributions on exposure variables, the comparison was limited to cases and controls residing within three miles of the specific industry type being evaluated. Each case could be identified with more than one industry and may appear in more than one analysis. Therefore, each industry study is a separate analysis, but not completely independent, from the others. The potential confounding from this source was limited by restricting the study to less urbanized parishes.

Exposure was defined as residence within 1.0 mile of an industry type; no exposure was defined as residence 1-3 miles from the industry under study. This arbitrary distance was selected because wind patterns were reported as too variable to define in this area and because particulate matter is

Table 1. Demographic comparison of cases and controls with verified length and location of residence.

Demographic characteristic	Cases		Controls	
	N	%	*N*	%
Age, years				
< 50	164	11.6	181	12.7
50–59	338	23.8	401	28.1
60–69	524	37.0	518	36.2
70	392	27.6	329	23.0
Race				
White	1113	78.5	1120	78.4
Other	305	21.5	309	21.6
Sex				
Male	1212	85.5	1222	85.5
Female	206	14.5	207	14.5
Year of death				
1960–63	175	12.3	172	12.0
1964–67	273	19.3	264	18.5
1968–71	391	27.6	421	29.5
1972–75	579	40.8	572	40.0
Total	1418	100.0	1429	100.0

reported to descend within one mile of exhaust depending upon the height of the stack, particle size, and density. Comparisons were limited to within 3 miles to control for the known urban-rural gradient in lung cancer mortality. This gradient is also associated with cigarette smoking, and socioeconomic status which was not examined in this study due to the already limited numbers available.

The same procedures were followed to obtain information for cases and controls. There were no significant differences in the numbers of located cases and controls by sex, race, age group, or parish.

Relative odds ($\widehat{RR}$) were calculated by using maximum likelihood point estimates, and the exact conditional confidence limits were computed for the unadjusted risk estimates (*23*).

Control of potential confounding variables and the modifying effect of others was obtained by using a multiple logistic analysis, (*24*). Standard errors of the parameters were calculated by use of the variance-covariance matrix, and standard errors of the adjusted relative risks were calculated from a linear combination of the appropriate matrix elements. Approximate 95% confidence limits of the adjusted relative risks were based on these standard errors.

Results

In all 3518 lung cancer deaths and 3518 control deaths were abstracted. Of these, information on residential location, and length at residence was available for 1418 (40.3%) cases, and 1429 (40.6%) controls.

Location and length of residence information was more complete on those who died during later years of the study, and more were male and white, compared to those with incomplete residence information.

The cases and controls with known residence information were compared on demographic characteristics using a log-linear model. Age was statistically associated with case-control status but differed on the average by only one year. This association was due to the priority system in matching on age, for which controls two years younger than the case were systematically sought first; mean age of the cases were 62.9 years and controls 61.7 years. Year of death, sex, and race were not associated with case-control status. Moreover, three way and higher order interactions were not found between age, race, sex, year of death and case-control status (Table 1).

Manufacturing industries identified as operating during the period 1942-1965 were classified into 30 different categories, which represented the complete study population. Those categories which either possessed a potential effect on the population based on earlier information, or had more than 40 individual plants in the category are shown in Table 2. These also represented most of the study population. The largest categories (> 100 individual plants) were lumber, grain, miscellaneous food, and printing. Those industry types with the largest average years in operation were paper (35.8 years), printing (33.8 years), chemical (30.8 years) and miscellaneous food (30.0 years). All industrial categories were in operation an average of more than 15 years, thus allowing an exposure period consistent with suspected latency periods for lung cancer. Only these

Table 2. Selected industries in operation between 1942 and 1965 in 20 Southern Louisiana parishes.

Industry type/(census code)	Specific industries (number)	Average years in operation
Lumber (107-9)	Logging, sawmills, miscellaneous wood products (163)	21.
Cement (127-37)	Cement, concrete, gypsum, plaster, clay, and pottery (65)	18.
Metal Mfg. (157-69)	Cuttery, hand tools, fabricated structural metal, screw machine, stamping and miscellaneous metal (63)	18.
Machine Mfg. (177-98, 258)	Machinery (except electrical), ordance (92)	28.
Food (268-9)	Meat, dairy products, (71)	25.
Canning (278)	Canning, preserving fruits, vegetables and seafood (84)	25.
Grain (279)	Grain-mill products (107)	23.
Bakery (287-9)	Bakery, confectionary, beverage and related products (89)	30.
Misc. Food (297-8)	Miscellaneous and not specified food (102)	30.
Paper (328-37)	Pulp, paper, paperboard mills and products (18)	35.
Printing (338-9)	Newspaper and allied publishing and printing (100)	33.
Chemical (347-9, 368-9)	Industrial chemicals, paints, varnishes, miscellaneous and not specified chemicals (67)	30.
Petroleum (49, 377-8, 387)	Crude petroleum gas extraction, petroleum refining, coal and miscellaneous plastic products (47)	17.1

13 industry types will be considered in the analysis below (Table 3).

For the following industry types, there was no evidence of any increase in risk associated with residential proximity to the industry: lumber, cement, machine manufacturing, printing and miscellaneous food (Table 2). The food and grain industries showed slight elevations in risk for 10 or more years exposure; the paper industry only in the case of large industries. However, for these risks 1.0 was included in the confidence intervals.

Though metal manufacturing and canning industries showed an excess risk for large industries, the sample size was too small to provide meaningful estimates. The petroleum industry showed elevations in risk for 10 years or more exposure. Examining the petroleum industry without consideration of industry size resulted in a relative risk of 1.65 (*RR* = 0.91).

The chemical industry showed elevations in risk with exposure to large industries, regardless of length of exposure, this was 2.11 and 1.51 for < 10 years and 10 + years exposure, respectively. When combined the risk was 1.81 (*RR* = 1.10).

The resulting residential risk estimates for grain, petroleum, paper and chemical industries after controlling for age, race, sex, year of death and birthplace by using a logistic model were similar to the uncontrolled risk estimates, with the petroleum and chemical industries showing the highest risk for 10 + years exposure and large industries, respectively. This is likely to be due to the effectiveness of the matching procedure.

The modifying effect of industry of occupation on the residential risk for the petroleum and chemical industries is shown in Table 4. Inclusion of industry of occupation as an effect modifier in the logistic model resulted in a significantly elevated residential risk only for those with a low risk industry of occupation, in those with residential exposure to large chemical industries ($\hat{RR}$ = 4.5, *RR* = 1.85) $\chi^2 = 12.4$, $p < 0.001$, not confined to more than 10 years exposure. An elevated although not significant, residential risk was noted in the low and high risk occupational groups for those with 10 or more years exposure to the petroleum industry (low risk RR = 1.34, RR = (0.41-4.330; high risk $\hat{RR}$ = 1.61, *RR* = (0.55-4.76)].

Except for proximity to large chemical industries, a comparison of overall residential and occupational risks revealed higher relative risk estimates for occupation than for residential exposure to either the petroleum or the chemical industry (Table 5). The residential risk was identifiable only for those with low risk occupations, and there was no evidence of synergism from exposure to both high risk occupation and residential proximity. Since the water source was controlled by matching on parish of residence, analysis of water source in cases and controls did not reveal any difference in potable water source, and an effect on residential risk by the type of potable water used (ground, surface) could not be studied. Exposure to more than one chemical or petroleum industry could not be validly evaluated due to small sample size of multiple exposures.

Discussion

This study describes a gradient of risk for lung cancer by length and/or amount of residential exposure for several selected industries, (food, canning, grain, paper, chemical and petroleum). As with any death certificate study, possible bias resulting from

Table 3. R ative risk estimates (RR) for lung cancer associated with known residential proximity to selected industry types by distance, size, and length of exposure

Industry type	Residential proximity, miles	< 100 employees						≥ 100 employees					
		0-9 yr exposure			≥ 10 yr exposure			0-9 yr exposure			≥10/yr exposure		
		Cases *N*	Controls *N*	RR	Cases *N*	Controls *N*	RR	Cases *N*	Controls *N*	RR	Cases *N*	Controls *N*	RR
Lumber	< 1.0	47	45	0.86	82	78	1.05	17	27	0.92	27	24	0.94
	≥ 1.0	34	28		26	26		11	16		12	10	
Cement	< 1.0	41	52	0.63	105	96	0.80	2	2	6.57	0	0	–
	≥ 1.0	44	35		37	27		7	4		1	1	
Metal Mfg.	< 1.0	26	34	0.58	81	82	0.87	8	10	1.08	1	2	–
	≥ 1.0	17	13		17	15		4	9		3	1	
Machine Mfg.	< 1.0	48	43	0.77	91	71	1.00	7	9	1.09	21	29	0.79
	≥ 1.0	32	22		36	28		5	7		11	12	
Food	< 1.0	55	71	1.06	93	116	1.30	6	10	0.30	57	64	1.29
	≥ 1.0	33	45		40	65		12	6		9	13	
Bakery	< 1.0	101	110	1.23	193	180	0.94	0	0	–	0	0	–
	≥ 1.0	62	83		50	44		0	0		0	0	
Miscellaneous Food	< 1.0	61	49	1.03	129	116	0.88	10	13	0.89	18	21	1.18
	≥ 1.0	46	38		38	30		13	15		21	29	
Printing	< 1.0	30	22	1.02	94	93	0.42	0	0	–	0	0	–
	≥ 1.0	8	6		17	7		0	0		0	0	
Canning	< 1.0	33	45	0.64	61	76	0.46	11	5	1.65	12	10	1.86
	≥ 1.0	15	13		28	16		8	6		5	7	
Grain	< 1.0	24	37	0.77	78	81	1.26	7	7	0.60	10	9	1.17
	≥ 1.0	21	25		13	17		15	9		19	20	
Paper	< 1.0	0	0	–	0	0	–	21	24	1.00	54	44	1.37
	≥ 1.0	1	1		0	0		27	31		60	67	
Chemical	< 1.0	24	31	0.60	20	20	0.69	16	11	2.11	55	35	1.51
	≥ 1.0	9	7		13	9		20	29		27	26	
Petroleum	< 1.0	11	16	0.63	11	5	1.47	17	17	0.68	32	24	1.65
	≥ 1.0	12	11		6	4		22	15		21	26	

Table 4. Modifying effect of industry of occupation, years of exposure or industry size on residential risk of lung cancer for selected industry types.[a]

Industry type	Industry of occupation	Exposure time, yr	Adjusted residential estimate	
		Industry size	$\hat{RR}$	*RR*
Petroleum[b]	Low risk	< 10 years	0.82	0.27–2.49
	Low risk[d]	≥ 10 years	1.34	0.41–4.33
	High risk	< 10 years	0.50	0.16–1.56
	High risk[e]	≥ 10 years	1.61	0.55–4.76
Chemical[c]	Low risk	< 100 employees	0.49	0.19– 1.26
	Low risk[f]	≥ 100 employees	4.54	1.85–11.11
	High risk	< 100 employees	1.33	0.27–6.47
	High risk	≥ 100 employees	0.82	0.34–2.00

[a] Exclud 21 cases and 18 controls exposed to chemical industry and 15 cases and 10 controls exposed to petroleum industry with unknown lustry of occupation.

[b] Contr d for years of death, age, race, sex, and industry size.

[c] Contro d for year of death, age, race, sex and years of exposure.

[d] Distan < 1.0 mile for 12 cases, 11 controls; distance ≥ 1.0 mile for 12 cases, 14 controls.

[e] Distan < 1.0 mile for 22 cases, 11 controls; distance ≥ 1.0 mile for 13 cases, 12 controls.

[f] Distan < 1.0 mile for 32 cases, 20 controls; distance ≥ 1.0 mile for 12 cases, 34 controls.

unmeasu ed exposures which may be related to distance rom industry may be present, and have not been evaluated. No cigarette smoking history was obta ned despite its known major contribution to lung ancer (*8-11*). However, the difference in observat ons resulting from the comparison of several industry types suggests that the major observed effect is not likely to be due solely to cigarette smoking. Other unknown environmental exposures were controlled by limiting case-control comparison to within three miles of the industry under study. However, the degree of control that was obtained

Table 5. Occupational and residential risk estimates for the chemical and petroleum industries for lung cancer.

	Industry of occupation[a]	Residential proximity	Predicted risk[b]		Cases	Controls
			$\hat{RR}$	*RR*		
Petroleum	+	+	2.27	1.04–4.92	36	23
	−	+	1.07	0.48–2.39	22	31
	+	−	2.52	1.12–5.66	34	20
	−	−	1.00		22	30
Chemical	+	+	2.56	1.27–5.16	41	26
	−	+	1.65	0.89–3.07	60	59
	+	−	2.72	1.27–5.81	33	19
	−	−	1.00		27	44

[a] Excludes 23 cases and 20 controls exposed to chemical industry and 18 cases and 14 controls exposed to petroleum industry with unknown industry of occupation.

[b] Controlled for year of death, age, race, years of exposure and industry size.

by this method is unknown. Nevertheless, this methodology was selected, as it enabled a sufficiently large geographic area without many confounding industries, to be surveyed over a sufficient time period to acquire enough subjects from areas with limited population in which to examine residential risks. Deaths due to other neoplasms were excluded in the controls to avoid confounding as other cancers might also be associated with exposure to a particular industry in question, and interfere with the observation of a true risk.

Approximately 40% of cases and controls had quantitative information on residence. As these subjects had only minor demographic differences in comparison to those subjects with incomplete residence information, generalization of the results to the population under study seems justified. Within the set of subjects with complete information, cases and controls were comparable in their distributions of race, sex, and years of death. The age difference of less than two years between cases and controls was not felt to be a biasing factor in the risk estimates for this disease.

The combination of industries into type is based on the similarity of their raw materials and products, and on the assumption of a similarity in the type of effluent produced. Exposure to industry by type is examined for each subject for the time period during which the subject was in residence and the industry in operation. Effluent measurements by industry were not available for the residential areas for the time periods of interest.

Residence in proximity to multiple industries should be examined to determine whether cause of death may have been preferentially related to proximity to a combination of industries, rather than to a specific industry type. A crude exposure index could be constructed to represent a function of distance of residence from the plant, length of exposure, and size and type of the industry. This could then be summed for all industrial exposures of a subject and used as a measure of total exposure to industrial effluents. This resulted in some positive results, but this approach precludes the identification, by type, of an industry particularly at high risk, and would be limited by the approximations used to combine industries by assuming similarities in products and effluents.

The duration of residence following exposure and prior to the onset of disease is unavailable from death certificate data. Date of diagnosis of disease is also unknown. For lung cancer, date of diagnosis and death are reasonable approximations of each other, and length of exposure to the time of death is an approximation of length of exposure to diagnosis. An assumption necessary for this study is that usual residence at death represents long-term residence at that address if it could have been validated. This assumption is warranted here due to the low mobility of the population in Louisiana, particularly in the parishes and age group selected for this study (1970 U.S. Census data).

The industries which did not produce elevated residential risk estimates, have not been reported to be associated with production of harmful effluents by others.

The cement and grain industries are known to release particulates (*25*). However, only for the grain industries was a slight elevation in residential risk, associated with 10 or more years exposure noted, and since only a small number of persons were available, no conclusions are warranted. In these parishes, grain industries have an average length of operation of five years longer than the cement industry. This suggests that the length of exposure to the cement industry may not be long enough to demonstrate a potential risk.

The slight elevation in residential risk for 10 or more years exposure to the food industry ($\hat{RR}$ = 1.3) is noteworthy, as a number of the industries in this category are involved in the smoking of meats, which may result in a release of polycyclic hydrocarbons.

The kraft paper mill industry, which predominates in the southern states, releases a variety of sulfides into the surrounding atmosphere (*26*). A moderate elevation in residential risk to this industry was noted for large industries with longer exposure. This is somewhat supportive of the association between lung cancer mortality and county employment in the paper industry found by Blot and Fraumeni (*17*).

The industry groups involved with metals include the metal manufacturing, machine manufacturing and canning industries. While certain categories tended to show elevations in residential risk, both their inconsistency with respect to a causal hypothesis (decreasing risk with increasing exposure), and small sample size reduced the epidemiologic significance of these observations.

While both occupational and residential risk to lung cancer has been shown for certain primary metal foundries and smelters, (*18*, *27*), none of these were represented among industries in this study.

The elevated residential risk to the petroleum industry (Table 3), for 10 or more years exposure, while not excluding 1.0 in the 95% confidence limit, is interesting in light of the variety and amount of hydrocarbons and gases released by these industries (*28*). The residential risk shown for this industry is shown for those with both low and high occupational risk, and long residential exposure (Table 4).

The strongest residential risk measured was for the chemical industry. Unlike that in the petroleum industry, the chemical industry risk was limited to large industries (approximately a twofold risk), regardless of length of exposure. A possible explanation for the differential risk in small and large industries is that only a limited number of the "small" industries were recorded as producing organic chemicals in the Directory of Manufacturers, while the majority of the "large" industries produce petrochemicals. Moreover, the aerial dispersion is likely to be more concentrated for "larger" industries. The observation of a residential risk to chemical industries at less than 10 years of exposure suggests that, in comparison to the petroleum effluents, a shorter period of exposure to these effluents may be sufficient for an effect.

The risk for residential exposure to the chemical industry was observed in those with only low risk occupational exposures. The 95% confidence interval of this 4.5-fold risk excluded 1.0. Therefore lower dose residential exposure for people in low risk occupations may result in a cumulative exposure to chemical carcinogens similar to that of the high risk occupational group. Since a further increase in risk is not noted for those already exposed to high risk occupations, additional low level exposure may not be additive or synergistic. Therefore, it is reasonable that this effect would only be observable in those without occupational exposures. It is also possible that the high and low risk occupational groups differ with respect to variables not measured, e.g., cigarette smoking, amount of time spent at residence and distance of residence from industry.

The observation that length of exposure was not as important in the chemical industry as in the petroleum industry suggests an hypothesis on the mechanism of carcinogenesis. It is possible that short-term damage from chemical industry exposure can induce a lasting risk of lung cancer (like asbestos), while for the petroleum industry longer exposure is necessary for accumulation of carcinogenic agents (more similar to cigarette smoking).

It is unlikely that the risks observed for the petroleum and chemical industries are due to other confounding exposures likely to be found in all major industrial agglomerations, as these observations were not found to be associated with the several additional industries which were similarly examined.

That these observations result solely from other undefined socioeconomic factors is unlikely, as there is no reason to believe that these factors would be specific only to certain selected industries. Since the population under study is limited to deaths, some degree of care must be used in extrapolation to the general population due to differential factors in migration due to illness. Further studies to validate these results and obtain the necessary socioeconomic data should be through next-of-kin contact. Furthermore specific studies on those at risk to these industries need to be undertaken.

The overall residential risk to chemical or petroleum industries was lower than that found for occupation (Table 5). However, as the number of exposed individuals was approximately twice as high for residence than for occupation, the resulting attributable risk percent for occupation and residence is equal, at approximately 3-4%.

Thus, the findings of this study suggest that residential proximity to the petroleum, and chemical industries may be an important risk factor for lung cancer in Louisiana. The consistency of the data and corroboration of other studies, in terms of both positive and negative findings by industry types, lend validity to the observations. The importance of residential risk to industries on a national level is unknown, but could be far more significant in terms of the numbers of exposed individuals than occupational exposure. Further research is required to validate the findings of this study, and to assess the true attributable risk percent, which is likely to be much higher than can be assessed through death certificate information where only the last usual residence is known, and information on other critical variables is unavailable.

This paper was presented at the 70th Annual Meeting of the American Association for Cancer Research, May 16 to 19, 1979, New Orleans, Louisiana. This work was supported in part by National Cancer Institute Contract No. 1 CP 61058, and Health and Human Resources Administration, the Department of Health, New Orleans, Louisiana.

Members of the staff who were highly dedicated to this study included J. Clarkson, H. Curcle, K. McGlynn and S. Weiner. Also, the contributions of W. Case, P. Ivy, R. Muller and B. Trahan are gratefully acknowledged.

Thanks are owed to the cooperation of the people of Louisiana who assisted in confirmation of residence and other factors without which this study could not have been accomplished.

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Environmental Health Perspectives
Vol. 45, pp. 165-170, 1982

Occurrence of Arsenic in Plaice (*Pleuronectes platessa*), Nature of Organo-Arsenic Compound Present and Its Excretion by Man

by J. B. Luten,* G. Riekwel-Booy* and A. Rauchbaar*

The arsenic content in 255 samples of plaice (*Pleuronectes platessa*) varied between 3 and 166 mg/kg. About 65% of the samples had an arsenic content above 10 mg/kg. High (low) arsenic concentration in the fillet corresponds with a high (low) concentration in milt or roe.

An excretion experiment with eight human volunteers showed that after the consumption of plaice, 69-85% of the ingested arsenic was excreted in the urine within five days. The organo-arsenic compound present in plaice was isolated by means of extraction, ion-exchange and thin-layer chromatography. Field desorption mass spectrometry of the isolate showed that arsenic was present as arsenobetaine, $(CH_3)_3\overset{+}{As}CH_2COO^-$.

Introduction

In general, high levels of arsenic are found in marine products. Chapman (*1*) observed high arsenic concentrations (2-132 mg/kg) among marine crustaceans and shell fish, whereas Schroeder and Balassa (*2*) reported arsenic levels ranging from 1.5 to 8.9 mg/kg in commercially available seafoods. High concentrations (2-38 mg/kg) in crabs were measured by Leblanc and Jackson (*3*), and concentrations varying between 0.15 and 18.7 mg/kg were found by Egaas and Braekkan (*4*) in 43 samples of fish products.

Westöö and Rydalv (*5*) determined the arsenic content of about 370 fish samples. Fish flesh, especially that from marine fish, and fish liver often showed arsenic levels above 1 mg/kg, the highest arsenic level being found in plaice (25 mg/kg). Various fish species have been analyzed for arsenic by Woidich and Pfannhauser (*6*). High concentrations were observed in conger eels (12-30 mg/kg), plaice (2-17 mg/kg), shrimp (0.4-12 mg/kg), sturgeon (0.7-12 mg/kg) and cod (2-10 mg/kg).

The World Health Organization (*7*) has published an acceptable daily intake of 50 μg arsenic/kg body weight. In view of the data given above, it appears that the consumption of marine products may be an important contribution to the total arsenic burden of human beings.

Chapman (*1*) showed that ~ 74% of arsenic (25 mg) present in lobster was excreted within 48 hr, whereas Coulson et al. (*8*) found that arsenic in shrimp was excreted completely within 4 days after consumption.

Schenk and Schreibeis (*9*) observed that the excretion of arsenic occurred mainly during the first 48 hr after consumption. Approximately 80% of the arsenic present in cod and plaice was excreted within 3 days (*5*). Munro (*10*), working with swine, observed that 68% of the arsenic from fish was excreted in the urine and 23% in the feces. Of arsenic from sole given to monkeys, 57-84% was excreted in the urine and 1-16% in the feces (*11*).

In 1977 Crecelius (*12*) reported the concentration of four different arsenic compounds [As^{3+}, As^{5+}, $CH_3AsO(OH)_2$ and $(CH_3)_2AsO.OH$] in urine after the ingestion of arsenic-rich substances (wine, well water, crabmeat). Arsenic present in wine as As^{3+} and As^{5+} was excreted 50% as dimethylarsinic acid, $(CH_3)_2AsO.OH$, 8% as As^{3+}, 8% as As^{5+} and 14% as

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methylarsonic acid [$CH_3AsO(OH)_2$]. It has been shown by Lakso and Peoples (*13*) and Tam et al. (*14*) that ingested inorganic arsenic will be metabolized as methylated arsenic. After the ingestion of crabmeat containing an unidentified organo-arsenic compound, the urinary concentration of the four arsenic compounds did not change significantly. However, when urine samples were digested with hot 2*N* NaOH, high concentrations of dimethylarsinic acid were present.

The behavior and toxicological properties of arsenic depends on the chemical form in which it is present. Penrose (*15*) mentioned that the toxicity decreases in the following way; arsines (trivalent inorganic or organic) > inorganic arsenite > arsenoxides > inorganic arsenate > pentavalent arsenicals > arsonium compounds (four organic groups with a positive charge on arsenic) > metallic arsenic.

Several investigators have made more or less successful attempts to elucidate the structure of the organo-arsenic compound(s) in marine fish. Lunde (*16*) and Brooke and Evans (*17*) reported the separation of inorganic arsenic (As^{3+} and As^{5+}) from organic bound arsenic in fish by distillation from hydrochloric acid. Although the distillation conditions were rather drastic, only small amounts of inorganic arsenic (0.01-2.5 mg/kg) were present.

Edmonds and Francesconi (*18*) showed that digestion of methanol extracts of mussels, western rock lobster and sting ray in aqueous sodium hydroxide led, after neutralization, to a solution from which the arsines, $(CH_3)_2AsH$ and $(CH_3)_3As$, were generated after reduction with sodium borohydride. The organo-arsenic compound isolated from western rock lobster by Edmonds et al. (*19*) has been identified as arsenobetaine, $(CH_3)_3\overset{+}{As}CH_2COO^-$.

Penrose et al. (*20*) isolated an organo-arsenic compound from witch flounder which possessed a mass spectrum that resembles tetramethylarsonium. The anion-exchange and thin-layer properties of the isolated compound, however, did not correspond with those of $(CH_3)_4\overset{+}{As}$. The structure of the isolated organo-arsenic compound from witch flounder has not been further elucidated.

Cannon et al. (*21*) reported the presence of arsenobetaine in dusky shark (*Carcharhinus obscurus*).

Kurosawa et al. (*22*) used HPLC and field desorption mass spectrometry for the identification of arsenobetaine in the muscle and liver of a shark (*Prionace glaucus*).

Recently, Edmonds and Francesconi (*23*) showed that the thin-layer properties of organo-arsenic isolated from school whiting are identical to those of arsenobetaine. It was therefore concluded that arsenic in school whiting is present as arsenobetaine.

Benson and Summons (*24*) mentioned that arsenic isolated from kidney tissue of a giant clam is mainly present as trimethylarsonium lactate and *O*-glycerophosphoryl trimethylarsonium lactate.

Still more information about arsenic in marine products is necessary. This paper describes an investigation into the occurrence of arsenic in an important flatfish species, plaice (*Pleuronectes platessa*), and gives some results of studies on the nature of an organo-arsenic compound present therein and the excretion by man.

Material and Methods

Sampling

During the period February 1979-March 1980, 255 samples of plaice (*Pleuronectes platessa*), caught at different fishing grounds in the North Sea, were obtained from the Netherlands Institute for Fishery Investigations, Ijmuiden, the Netherlands. The edible parts, liver, milt and roe, were homogenized separately and stored at -20°C until the analysis. Table 1 presents a survey of the sampling data.

Determination of Arsenic

Total Arsenic. The homogenized samples were dry-ashed overnight at 550°C in the presence of $Mg(NO_3)_2$ and MgO. After dissolving the ash in hydrochloric acid, As^{5+} was reduced to As^{3+} with the addition of potassium iodide.

Arsine, formed by the use of zinc-hydrochloric acid as hydride generator, was absorbed in a solution of silver diethyldithiocarbamate in pyridine. The absorbance at 528 nm was then measured.

Methylated Arsenic. A homogenized fish sample was submitted to a Bligh and Dyer extraction (*25*). After evaporation of methanol from the aqueous/ methanol phase at 40°C, the aqueous fraction was digested in hot 4*N* KOH for 2 hr. In the case of urine samples, the Bligh/Dyer extraction procedure was omitted. After the KOH digestion the sample could be analyzed for inorganic and methylated arsenic by reduction with sodium borohydride at $pH < 1$. During the reduction procedure the hydrogen and the arsines formed were collected in a 2-liter bottle filled with deionized water which was placed upside down in a water bath. The collection was terminated after 2 min and the bottle was then closed under water with a septum. In preliminary experiments it was established that under these conditions the arsenic present was recovered completely. A 1 ml gas sample was submitted to gas chromatography. Detection was performed with a

Table 1. Concentration of arsenic in plaice *(Pleuronectes platessa)*.

Date	Number of samples	Arsenic concentration, mg/kg		
		Average	Range	Median
790215	10	48	9–166	24
790301	10	29	18–49	27
790504	21	15	4–70	11
790522	20	16	8–35	14
780615	20	11	3–19	13
790713	20	57	15–119	60
790907	20	14	4–40	12
790927	20	11	6–21	11
791101	16	15	5–31	12
791129	20	11	3–53	7
791221	18	19	5–77	10
800117	20	28	3–97	19
800222	20	24	8–86	15
800327	20	41	6–144	34
Total sampling period	255	23	3–166	14

microwave-induced plasma detector. The conditions used for the separation of AsH_3, CH_3AsH_2, $(CH_3)_2AsH$ and $(CH_3)_3As$ were as follows. Glass column, 1.8 m × 1.8 mm ID packed with 5% Carbowax 20M on Chromosorb 101 (80-100 mesh); column temperature, 170°C; injection port temperature, 200°C; wavelength, 235.0 nm; split width, 0.2 nm; microwave generator output, 75 W; carrier gas, He; capillary pressure, 50 mm Hg. Solutions of As_2O_3, $CH_3AsO(ONa)_2$, $(CH_3)_2AsO.ONa$ and $(CH_3)_3AsCl_2$ were used as standards.

Isolation Procedure for Organo-Arsenic Found in Plaice

The isolation procedure for the organo-arsenic compound found in plaice consisted of three steps; extraction, ion-exchange and thin-layer chromatography.

About 210 of plaice (166 mg As/kg) was subjected to a lipid extraction according to the Bligh and Dyer procedure (*25*). Methanol was evaporated from the methanol aqueous phase at 40°C. The aqueous extract, acidified by adding HCl until a pH 3 was reached, was then passed through a cation-exchange column (Amberlite IR-120, 20-50 mesh, H^+ form). Elution of the retained organo-arsenic compound was performed with 2% NH_4OH. After neutralization, the eluate was applied to a mixed-bed column (Amberlite, IRA-400, 20-50 mesh, OH^- form, Amberlite IRC-50, H^+ form). The eluate, which contained the organo-arsenic compound, was subjected once again to the cation-exchange procedure. Fractions of the NH_4OH elution step containing the arsenic-compound were then collected. The overall recovery of arsenic from plaice by the described isolation procedure was about 80%.

Thin-layer chromatography of the isolate on cellulose with *n*-butanol/acetic acid/water (60:15:25) resulted in three spots (R_f = 0.65, 0.58 and 0.48) after exposure to iodine vapor. Only the spot with R_f = 0.58 contained arsenic. This arsenic-containing spot was then extracted with methanol and subjected to field desorption mass spectrometry.

Excretion Experiment

Eight healthy human volunteers (6 male, 2 female) each consumed 100 g plaice (80 mg As/kg). Prior to this experiment none of the participants had eaten arsenic-rich food. During the first 12 hr of the experiment the volunteers were asked to urinate at 2 hr time intervals. After 5 days the experiment was terminated. All urine samples were analyzed for the presence of arsenic according to the procedure described above for methylated arsenic.

Results and Discussion

The average and median concentrations and the concentration ranges of arsenic in fillets of plaice are given in Table 1. A frequency distribution of arsenic over the 255 investigated samples of plaice is given in Figure 1. A large variation in the arsenic content was observed. About 65% of the samples had an arsenic content above 10 mg/kg.

The arsenic content in the fillet, liver, milt and roe of 18 samples of plaice (Table 2) show that a high (low) arsenic content in the fillet corresponds to a high (low) arsenic level in the liver and milt. In some liver samples the arsenic content was several times higher than in the corresponding fillets. It is not clear to what extent this was due to accumula-

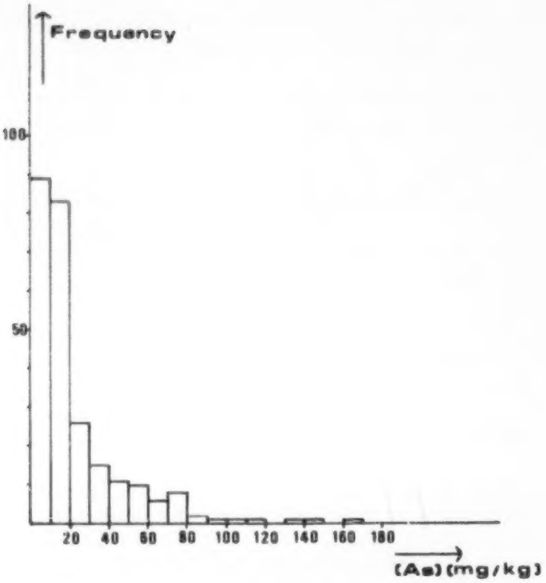

FIGURE 1. Frequency distribution of arsenic in 255 samples of plaice.

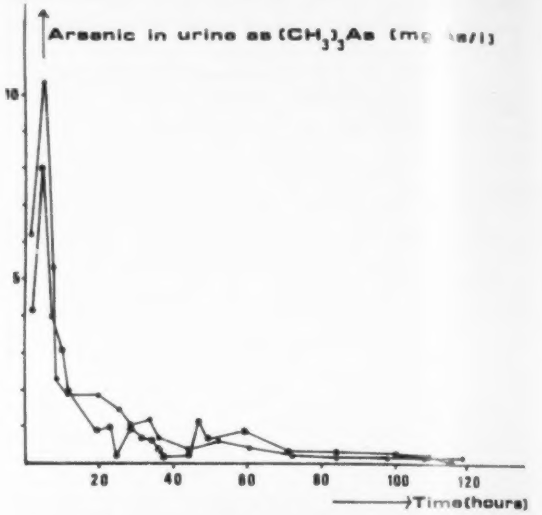

FIGURE 2. Concentration of arsenic in urine, as $(CH_3)_3As$, as a function of time after the ingestion of plaice by two human volunteers. The urine samples were analyzed for the presence of arsenic following the procedure described in the section on methylated arsenic.

tion of arsenic in the liver. Compared with the fillets, the arsenic content in roe is low.

The excretion of arsenic in the urine of eight human volunteers after 5 days varied between 69 and 85% (average 75%) of the administered dose. As an illustration the arsenic content in the urine of two volunteers is given as a function of the time in Figure 2. The bulk of the ingested arsenic was excreted during the first 24 hr after consumption.

Only small amounts of inorganic arsenic (< 30 ng/ml) were present in the urine samples prior to the excretion experiment. All the arsenic excreted after consumption of plaice was present as trimethylarsine in urine after alkaline digestion and reduction with $NaBH_4$, at low pH. Other forms of methylated arsenic as well as inorganic arsenic

Table 2. Arsenic content in fillet, liver, milt or roe of 18 samples of plaice.

Sample	Sex	Arsenic concentration, mg/kg			
		Fillet	Liver	Milt	Roe
1	F	135	318	–	5
2	F	29	26	–	[illegible]
3	F	60	53	–	[illegible]
4	F	47	92	–	3
5	F	97	89	–	9
6	F	86	431	–	14
7	F	34	126	–	8
8	M	77	44	42	
9	M	76	199	65	–
10	M	57	75	43	
11	M	103	83	28	
12	M	73	44	72	
13	M	5.3	2.4	2.6	
14	M	5.4	1.3	2.3	–
15	M	5.8	2.3	1.9	–
16	M	6.1	2.8	2.5	
17	M	5.8	1.5	2.8	–
18	M	5.9	3.6	2.7	–

Table 3. Some properties of organo-arsenic compounds.

Compoun	After the alkaline digestion/reduction procedure	R_f (BuOH/HAC/H_2O)
Arsenobe ine, $(CH_3)_3\overset{+}{As}CH_2COO^-$	$(CH_3)_3As$	0.58
Arsenoch ne, $(CH_3)_3\overset{+}{As}CH_2CH_2OH$	Trace $(CH_3)_3As$	0.58
Tetramet larsonium iodide, $(CH_3)_4As^+I^-$	—	0.35
Organo-a enic from plaice	$(CH_3)_3As$	0.58

were not present. It was established that no volatile arsines were present if the alkaline digestion is omitted prior the reduction procedure. The necessity of an alkaline digestion prior to the reduction procedure agrees with the results of Crecelius (*12*). However, in this investigation trimethylarsine is formed in urine after reduction, while Crecelius reported the formation of dimethylarsine.

It was demonstrated that the bulk of the arsenic in plaice (95%) was present in the aqueous fraction after the Bligh and Dyer extraction. Digestion with KOH followed by a reduction with $NaBH_4$ led to the formation of $(CH_3)_3As$. The properties of the organo-arsenic reference compounds after the digestion procedure as well as the R_f values, given in Table 3, indicate that the isolated organo-arsenic compound found in plaice was neither arsenocholine nor tetramethylarsonium.

The mass spectra obtained by field desorption mass spectrometry of the isolated organo-arsenic compound, arsenobetaine and a mixture of the two shown in Figure 3. According to the molecular structure of arsenobetaine the protonated molecular-ion $(CH_3)_3(\overset{+}{As}CH_2COOH$ ($m/e = 179$) and two fragments $(CH_3)_4\overset{+}{As}$ ($m/e = 135$) and $(CH_3)_3\overset{+}{As} + \overset{\cdot}{C}H_2$ ($m/e = 134$) are present. The peak at $m/e = 134$ was the base peak. The mass spectrum of the isolated organo-arsenic compound showed the presence of an ion at $m/e = 135$ as the base peak and an ion at $m/e = 179$ with small intensity. No peak at $m/e = 134$ was present. The addition of an equivalent amount of arsenobetaine to the isolated compound caused a significant shift in the fragmentation pattern of pure arsenobetaine. The nominal mass $m/e = 135$ became the base peak, while the relative intensities of $m/e = 134$ and $m/e = 179$ decreased considerably. Protonization of arsenobetaine, probably due to impurities present in the isolated compound, increased the presence of the tetramethylarsonium ion.

High resolution field desorption mass spectrometry ($R = 10^4$, 10% valley definition) showed that the exact mass of $m/e = 179$ of arsenobetaine and that of the isolated organo-arsenic compound did not

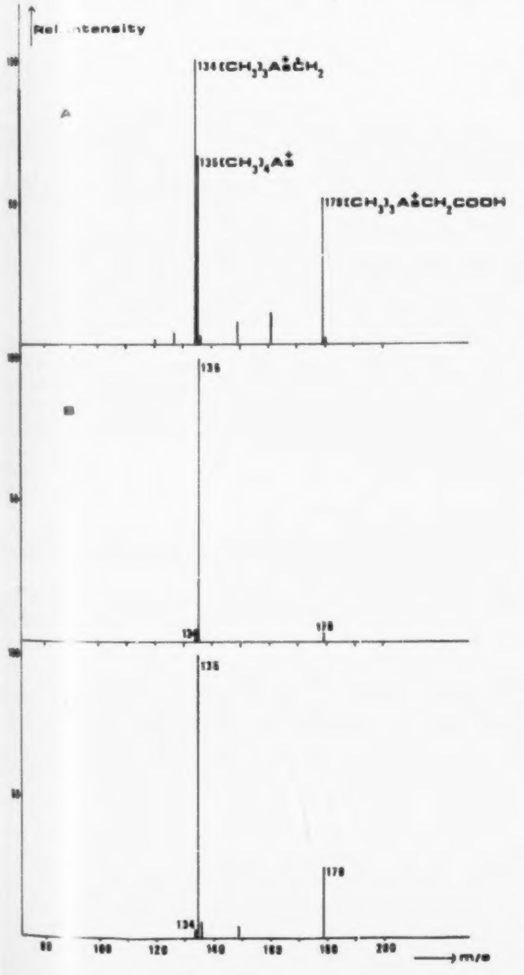

FIGURE 3 Field desorption mass spectra of (*A*) arsenobetaine, (*B*) the organo-arsenic compound from plaice and (*C*) a mixture of the two.

differ significantly from each other. It was therefore concluded that the arsenic found in plaice is present as arsenobetaine.

The authors are grateful to J. van de Greef (CIVO-Analysis TNO) for his help in the field desorption mass spectrometry and to H. Meinema (OCI-TNO) in the synthesis of the organo-arsenic compounds. This research was supported in part by the Ministry of Agriculture and Fisheries, the Hague, the Netherlands.

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Environmental Health Perspectives
Vol. 45, pp. 171-182, 1982

Risk Assessment in a Federal Regulatory Agency: An Assessment of Risk Associated with the Human Consumption of Some Species of Fish Contaminated with Polychlorinated Biphenyls (PCBs)

by Frank Cordle,* Raymond Locke,*† and Janet Springer*†

The problem of polychlorinated biphenyls (PCBs) became a national concern in 1971 when several accidental contaminations of foods were reported. Extensive efforts were undertaken by FDA to reduce the residues of PCBs in food. However, the PCB levels in several species of fresh-water fish have raised concern about the PCB residues from environmental contamination, and it is this concern which has prompted a reassessment of the human risk involved from consumption of such fish. The human epidemiology and animal toxicity of PCB exposure are reviewed, as well as risk assessment in general. Specific examples of risk assessment involving extrapolation of animal data to humans, based on several levels of human exposure to PCBs in fish, are presented.

Introduction

Perhaps the most important issue in policy decisions relating to toxic substances in the environment is an assessment of the human risk from exposure to such toxic substances. The best evidence that an agent is toxic to humans or is a human carcinogen with subsequent human risk that can be quantified is provided by adequate epidemiology data backed by confirmatory animal data. However, for practical purposes, most decisions on human toxicity and carcinogenicity are based on animal studies. To a large degree, the problems involving risk to human health from exposure to polychlorinated biphenyls (PCBs) in some species of fish require extrapolation to humans with all of the uncertainties inherent in such methods.

The term PCBs refers to a complex mixture of different chlorobiphenyls and isomers. Isomers are two compounds which have the same number of chlorine substitutents on the biphenyl molecule but at different locations.

PCBs were reportedly first synthesized in 1881 but were not commercially available until 1930 (*1*). The only domestic producer was the Monsanto Company. The use of PCBs in a wide variety of industrial applications steadily increased from 1930 until 1971, when the manufacturer voluntarily restricted their distribution in the U.S. to closed systems, including electrical transformers, capacitors, and heat transformers.

PCB products manufactured by Monsanto in the U.S. are identified by the trade name Aroclor, and the particular kind of Aroclor is identified by a four-digit number, e.g., Aroclor 1254 or Aroclor 1260. The first two digits refer to the 12 carbon atoms that make up the biphenyl and the second

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two digits refer to the approximate content, in weight percentage, of chlorine in the mixture. Under this numbering system, Aroclor 1254 contains 12 carbon atoms and about 54% chlorine, while Aroclor 1260 contains 12 carbon atoms and about 60% chlorine.

PCBs became a national concern in 1971, when several incidents of accidental contamination of foods were reported. In addition, the extent of the environmental contamination and its persistence were not precisely known. Subsequently, various regulatory actions were taken by the agencies involved, and with the cooperation of the only U.S. producer, the situation was felt to be under control.

Then, in 1975, with the reported high levels of PCB contamination in Hudson River fish, national attention was refocused on PCBs. It was soon apparent that the actions and control measures of the early 1970s had not succeeded in totally reducing or even substantially alleviating the problems associated with PCB contamination in the environment.

Despite the many industrial applications of PCBs in the U.S. for 40 years, information on human exposure and on adverse human health effects from such exposure in the U.S. is scant. In this country, sources of minimal human exposure of the population to PCBs are limited to food, air, and water; perhaps the most significant human exposures are limited to sports fishermen consuming fresh-water fish from contaminated streams and lakes, and to occupational exposure in industrial workers. However, the PCB levels in several species of fish have raised concern about the PCB residues from environmental contamination, and these concerns have prompted a reassessment of the PCB action levels. A typical PCB residue from fish resembles the Aroclor 1254 mixture more closely than it does other Aroclors (*2*, *3*).

A review of the data on fish residues gathered by various agencies indicates a serious incompatibility among sampling programs for obtaining data that reliably define trends in PCB levels. The Food and Drug Administration's primary concern is the levels in the edible portion of commercially important fish marketed interstate. Therefore, in most cases, the heads, entrails, skin, and fins are excluded. Furthermore, FDA has not generally sampled fish that are caught and consumed locally, and the sampling usually has dealt with the most important consumption species, which are primarily of marine origin and generally do not contain as high PCB levels as fresh-water species from certain locations. An FDA 1978-1979 survey (unpublished) of PCB residues in fish, including fresh-water species, presented some evidence that fresh-water fish continue to be the major source of igh PCB residues in the food supply.

To determine human exposure to PC through dietary fish, one must know the lev of PCB residues in the edible portions of fis and the amounts of the various kinds of fish c umed by the population at large and by special s groups of the population. Information has been npiled by the National Marine Fisheries Servic OAA (*4*) on the most important types of fish e en in the U.S. today and the mean daily amount each type eaten by the subpopulations of actual ers. This study included a sample of 25,947 h eaters selected to represent all fish eaters in e United States.

Information from the survey indicate that some 20 species comprise 95% of all the fish products eaten. Although 93% of the U.S. population (197 million) eat fish, the average annual per capita consumption is small: 15.0 lb/year, with major consumption of a large "unclassified" fish fraction that exists in the U.S. diet, ranking just below tuna in importance. This "unclassified" fraction of fish represents a variety of fish species, each of which, taken separately, would contribute only a minor proportion of the diet. However, when taken as a group, those species represent a major contribution to fish consumption in the U.S. The major portion of many of our most familiar types of seafood is imported, and fresh-water species, led by trout, bass, and catfish, comprise about 9% of our total fish diet.

Table 1 describes the 12 species of interest, i.e., those species of fish found in the FDA 1978-1979 survey to have the highest PCB residu levels as well as the mean PCB levels in these spe ies. Table 2 describes the daily intake of PCBs in th se people who ate the 12 species of interest (3939 dividuals out of the total of 25,947) at the 50t and 90th percentiles.

Epidemiology of Human Ex osure

Considerable scientific interest has c tered on the Yusho incident in Japan. In 19 , human intoxication with Kanechlor 400, a PCB ma ıfactured in Japan, was noted when a heat exchan r leaked this PCB into rice oil ("Yusho" oil) hat was consumed by Japanese families.

The typical clinical findings included hloracne and increased pigmentation of the skin, ncreased eye discharge, transient visual disturbances, feeling of weakness, numbness in limbs, headaches and disturbances in liver function. Most of the babies born to affected mothers had skin discoloration

Table 1. Mean PCB levels in FDA 1978–1979 domestic survey by species of interest.[a]

	Assumed tolerance = 0[b]		Assumed tolerance = 5 ppm		Assumed tolerance = 2 ppm		Assumed tolerance = 1 ppm	
Species of interest	Mean PCB, ppm	N	Mean PCB, ppm	N	Mean PCB, ppm	N	Mean PCB, ppm	N
Carp	1.10	54	0.90	52	0.68	46	0.54	38
Catfish	1.70	295	1.19	281	0.73	219	0.38	150
Buffalo	0.50	36	0.50	36	0.43	35	0.30	31
Fresh water trout	1.36	87	1.28	85	0.76	58	0.37	40
Sea trout	0.56	10	0.56	10	0.56	10	0.27	8
Bass	1.28	15	1.28	15	0.77	11	0.27	10
Chubs	1.14	19	1.14	19	0.96	17	0.58	9
Bluefish	0.53	23	0.53	23	0.44	22	0.37	20
Scup (porgy)	0.72	10	0.72	10	0.72	10	0.53	8
Drum	0.49	12	0.49	12	0.49	12	0.32	10
Mackerel	0.53	21	0.53	21	0.53	21	0.28	17
All others	0.26	206	0.26	206	0.24	204	0.22	201

[a] Tuna and shellfish were assumed to have 0.0 level of PCB. For assumed tolerance, PCB values below the tolerance were eliminated in calculating the mean.

[b] No tolerance.

Table 2. Intake of PCBs from fish for eaters of species of interest (3939/25,947).[a]

	Intake at 50th percentile			Intake at 90th percentile		
Assumed tolerance, ppm	µg per day	ppm of diet[b]	µg/kg body weight[c]	µg per day	ppm of diet[b]	µg/kg body weight[c]
0[d]	8.46	0.0056	0.72	22.1	0.0147	0.32
5	7.57	0.0051	0.11	20.3	0.0135	0.29
2	5.59	0.0037	0.08	14.9	0.0099	0.21
1	3.30	0.0022	0.05	9.22	0.0061	0.13

[a] For assumed tolerance, PCB values below the tolerance were eliminated.

[b] Assumed 1500 g daily intake.

[c] Assuming body weight of 70 kg.

[d] No tolerance.

which slowly regressed as the children grew. Adult patients had protracted clinical disease with a slow regression of symptoms and signs, suggesting a slow metabolism and excretion of the PCB in humans probably due to a long biological half-life.

A review of the literature extant in 1972 revealed the following facts. The average PCB content of the rice oil in the dose-response epidemiologic study was 250 ppm. In this study, the average cumulative intake of PCBs leading to overt symptomatology was 20 mg, and the lowest dose leading to overt symptomatology was 500 mg. Originally, rice oil contaminated with a heat exchanger, Kanechlor 400, a polychlorinated biphenyl, was associated with Yusho symptomatology. PCBs were identified in the contaminated rice oil consumed and in the blood and tissues of patients. Therefore, the effects seen were attributed to PCBs.

In the review by Kuratsune et al. (*5*) a new factor was introduced into the system; namely, the canned rice oil was also contaminated with chlorinated dibenzofurans (PCDFs) to the extent of 5 ppm. In addition, in this same paper Kuratsune presented data of Nagayama et al. (*6*) showing polychlorinated dibenzofurans to be present in the liver and adipose tissue of Yusho patients, while none was found in that of a control group. Nagayama et al. (*6*) reported that the ratios of PCBs to PCDFs in Kanechlor 400, in a Yusho oil of February 5 or 6, 1968, in adipose tissue, and in liver from a patient were 50,000, 200, 144 and 4 to 1, respectively. Thus, relative to Yusho oil, the liver with a PCB/PCDF ratio of 4 to 1 appears to concentrate PCDFs selectively relative to the PCBs. If PCDF is 200 to 500 times more toxic than PCB, the contaminated rice oil would be 2 to 3.5 times more toxic than expected from the PCB content alone. Additional work has indicated that the original estimate of PCBs in the rice oil was in error because the analysis was based on total organic chlorine present, and that the rice oil contained approximately 1000 ppm of chlorinated quaterphenyls in addition to the PCB residues. A discussion of the use of these data in attempting to set so-called safe levels of PCBs in the diet will be presented in a later section of this report.

The earliest reports of adverse health effects due to exposure of workers to PCBs in this country are probably those of Schwartz (*7*), who described skin lesions and symptoms of systemic poisoning among workers who were said to have inhaled chlorobiphenyls; their complaints included digestive disturbances, burning of the eyes, impotence and hematuria. Patch tests with the chlorobiphenyls were negative, and Schwartz speculated that mechanical plugging of the follicles of the skin as the fumes solidified on it was responsible for the skin lesions. The chlorine present in the products was thought to then exert an irritating effect on the plugged follicles and to thus cause suppuration. No quantitative data were reported, but a number of preventive practices were recommended.

There have been numerous reports over the ensuing years (*1*) of cutaneous eruptions and of systemic manifestations as well, among marine electricians, machinists, capacitor and transformer manufacturing workers, and other occupationally exposed to PCBs. However, in many of these reports the exposures are described as having been to mixtures of chlorinated hydrocarbons, quite often of chlorinated naphthalenes and PCBs.

The skin lesions described by Schwartz (*7*) have come to be designated generally as chloracne. Chloracne can be produced by a number of chemical compounds, including chlorinated dibenzofurans and certain isomers of the chlorinated dibenzodioxins (*8*). Oily skin and large pores seem to predispose to the disease, while the opposite is the case for smooth, tender skin.

Part of the chloracne lesion resembles adolescent acne, but it is generally more severe, and lesion distribution is inconsistent with adolescent acne although it may be superimposed upon it. It is known that chloracne can be produced by either the systemic absorption of chlorinated biphenyls or the direct application of chloracnegenic compounds to the skin. Systemic effects sometimes result after occupational chloracne has manifested itself; these may include loss of appetite, nausea, edema of the face and hands, abdominal pain, vomiting and burning and soreness of the eyes. No fully satisfactory explanations have been made of the development of chloracne. Chloracne is generally very persistent, and there is no preferred control measure.

As indicated previously, low levels of human exposure to PCBs in the U.S. population may occur from air and water. Samples of ambient air were collected in suburban areas of Miami, Florida; Jackson, Mississippi; and Fort Collins, Colorado. Preliminary results (*9*) for samples taken in April, May and June of 1975 show that PCBs were present at all locations. Although the data varied, the average concentrations at each of the three locations was approximately 100 ng/m^3. Initial identification of the PCBs indicated that they were most comparable to the Aroclor 1254 standard.

Dennis (*10*) has also reported that data gathered from monitoring activities of surface waters and bottom sediments of the major drainage basins of the United States indicate the widespread occurrence of PCBs in both surface water and bottom deposits. A preliminary assessment of PCB levels shows mean residue levels in water ranging from 0.01 to 0.05 μg/l. The 0.05 μg/l levels were found in the south Atlantic slope and eastern Gulf of Mexico drainage basin. In general, the lowest PCB residue levels were found in drainage basins west of the Mississippi.

The Great Lakes represent another area of the nation contaminated with PCBs. The Michigan Water Resources Commission (*11*) reports that many surface water samples contained PCBs at concentrations above the detection limit of 10 parts per trillion. In this study, residues of PCBs were found at 10 locations from rivers and streams discharging into the Great Lakes. Effluents from wastewater treatment plants servicing industrialized communities have been found to be highly contaminated.

Sampling surveys of Great Lakes fish have shown that most species tested contained detectable levels of PCBs and that residue levels were generally proportional to fish size (age) and were highest in the predator species. Except for whitefish, the species of commercial or sport interest (trout and salmon) from Lake Michigan were found to be highly contaminated with PCBs. Data obtained from lake trout collected from various areas of Lake Michigan show mean PCB levels ranging from 3.06 ppm to 11.93 ppm.

The Michigan Department of Public Health has recently completed a study (*11*) which attempted to assess some of the consequences of human exposure to PCBs from the consumption of sportsfish caught in different areas of Lake Michigan. The study included exposed and control subjects from five areas of Michigan bordering on Lake Michigan. Exposed study subjects were those individuals who consumed at least 24 to 26 lb of Great Lakes fish per year. Control subjects were those individuals who consumed less than 6 lb of Great Lakes fish per year.

An assessment of the findings in the study indicates that the most frequently recorded quantity of fish consumed by the study participants was in the 24-25 lb/year range. The highest recorded fish consumption over the two-year period of the study was 180 lb/year and the highest single-season consum tion as 260 lb. Mean PCB levels in whole lake tr t are reported as 18.93 ppm in 1973 and 22.91 p n in 1974, and as 12.17 ppm in Coho salmon in 1973 d 10.45 ppm in 1974. However, comparisons of PCB evels in raw versus cooked fish indicated that ac al human exposure to PCBs from fish consum ion is less than might be expected from the raw fish data. This is not unexpected, since prepar on (trimming away fatty tissue) and cooking have re ulted in a decrease in the amount of PCBs actually consumed in fish. For example, the PCB level in cooked lake trout consumed by the study partici nts ranged from 1.03 to 4.67 ppm; in cooked almon from 0.48 to 5.38 ppm; and in other cooked sh from 0.36 ppm to 2.06 ppm. These levels are de dedly lower than the level of PCB contaminat on reported in raw trout and salmon.

PCBs were found in all blood specimens collected from the 182 study participants during the study period, including controls. The values ranged from a low of 0.007 ppm in blood in the control group to a high of 0.366 ppm in the exposed group. Although there was a wide range of blood values for each quantity of fish consumed, there was a positive correlation between the reported quantity of Lake Michigan fish consumed and the concentration of PCB in the blood of study participants. No annual variation in PCB blood levels in humans could be demonstrated. The mean PCB blood values for the control and exposed groups did not appear to change markedly from 1973 to 1974. In addition, abstinence from Lake Michigan fish consumption for a period of 90 days or more did not change the PCB blood levels significantly. PCB blood levels over the abstaining period show variation but no steady decline in PCB. In fact, more subjects showed no change or a rise than showed a decline in PCB blood levels over time.

The calculated quantity of PCBs ingested by eating Lake Michigan fish averaged 46.5 mg/year and ra ged from 14.17 to 114.31 mg/year. PCB ingesti n for each individual was determined by propor oning his/her reported annual fish consumption b frequency of species eaten and the cooked fish P B levels for those fish. The community averag for cooked fish was used in instances in which ooked fish determination was not available for st ly participants. Because fish consumption was fo nd to vary from year to year, the average annua consumption for each individual for the two baseli years of study was used in each case.

Res ts from this study indicate that the calculated mean aily dose received by the exposed group is 1.7 μ kg/day and ranges from 0.09 to 3.94 μg/kg/da If the average annual rate of PCB ingestion from sh indicated by these study results were contin ed over the years, the average sports fisherman consuming contaminated fish could receive a total PCB dose equal to 200 mg in approximately 4.3 years. Under the same set of assumptions, individuals consuming greater than average amounts of contaminated fish would reach the total dose level sooner. No adverse health effects or groups of symptoms that were clearly related to PCB exposure could be identified in the exposed group. This implies that exposure to PCBs from eating contaminated fish at the levels observed and the presence of PCBs in these exposed persons have not caused any observable adverse health effects similar to those observed in the Yusho population. However, this does not exclude the possibility that effects too subtle for detection are occurring, or the possibility of long-term health effects. Because of the lack of sufficient human data, risk assessments for potential toxic effects of chemicals must of necessity be estimated from animal experiments. In the absence of contradictory kinetic or metabolic data, the animal data are used to estimate potential human risks. Because the numbers of animals used in tests are limited, doses above the human exposure levels are used to increase the probability of detecting potentially toxic chemicals.

Thus, the risks at low doses must be estimated from higher experimental doses. Because of the inability to observe the low end of the dose response curve with precision, the linear (or when necessary one-hit) extrapolation from high to low doses is often used (*12-15*). Because of the many uncertainties involved in risk estimation, this approach is conservative and errs in favor of public health. Also, linear (one-hit) extrapolation is the limiting case for the multistage model of carcinogenesis at low doses. Since the shapes of dose-response curves at low doses are unknown, it must be remembered that actual estimates of risk are not being obtained. Based upon plausible assumptions, however, it generally is possible to place upper bounds on potential risk by use of linear (one-hit) extrapolation based upon animal data.

Animal Data

Several elements related to risk must be considered in any assessment of animal data: for example, the similarity of exposure in animals compared to humans, e.g., types of Aroclors used in animal experimentations (most human exposure is to Aroclor 1254, and a typical residue from fish resembles the Aroclor 1254 mixture more closely than it does the other Aroclors) (*2*, *3*), and the kinds of outcome that might be comparable to those for humans. Risk assessment—or the estimation of the acceptable daily intake—will be based on general toxicity, carcinogenicity and the effects on reproduction for which there is little or no previous experience in such risk assessment.

The acute toxicity (oral and dermal) of the PCBs is of relatively low order when the substances are administered as a single dose. In contrast, the subacute toxicity of both the PCBs and individual chlorinated biphenyls appears to be of far greater concern; species sensitivity and cumulative toxic effects appear after continuous exposure at low levels (*16-18*). The effects of various PCB compounds have been studied in a number of animal species, and the results have been compiled and evaluated in recent reviews (*1*, *19*). In assessing the possible toxicological hazard posed by PCBs for humans, it is preferable to utilize animal feeding studies in which the PCBs are added at low levels to the diet and the treatment is continued essentially throughout the life span of the animals. Few such long-term studies of the toxicity of PCBs are currently available. For assessing the risk posed to humans as a result of exposure to PCBs, three long-term studies have been chosen which are cited and discussed in detail below: the National Cancer Institute's bioassay of Aroclor 1254 for possible carcinogenicity in male and female Fischer 344 rats (*20*), the study of Kimbrough et al. (*21*) on the induction of liver tumors in Sherman strain female rats by Aroclor 1260, and the 11-month study by Kimbrough and Linder (*16*) of the toxic effects of Aroclor 1254 in male BALB/cJ mice. In addition, because of the known extreme sensitivity to PCB-related toxicity of the rhesus monkey compared to rodent species, the short-term study by Allen and Norback (*22*) of the pathological responses of these primates to PCB exposure as a basis for risk assessment is also described.

In the National Cancer Institute's bioassay of Aroclor 1254 (*20*), groups of male and female Fischer 344 rats (24 of each sex per group) were administered the test compound in the diet at 25, 50 or 100 ppm levels for a period of 104-105 weeks. Matched controls consisted of groups of 24 untreated rats of each sex. All animals were observed daily for signs of toxicity and palpated for tissue masses at each weighing. Moribund animals were sacrificed and subjected to gross and microscopic pathological examination, as were the animals sacrificed at the end of the experimental period. It was concluded that, under the conditions of the bioassay, Aroclor 1254 was not carcinogenic in Fischer 344 rats; however, it was suggested that the high incidences of hepatocellular proliferative lesions of the gastrointestinal tract in the Aroclor-treated males and females might be associated with the administration of the compound.

In the study by Kimbrough et al. (*21*), 200 Sherman strain female rats were fed a diet containing 100 ppm of Aroclor 1260 for approximately 21 months, and treatment was discontinued for 6 weeks before the animals were sacrificed at 23 months. A group of 200 untreated female rats served as controls. All animals were observed daily, and moribund animals were sacrificed and subjected to gross and microscopic pathological examination, as were the animals sacrificed at the end of the experimental period. The authors concluded that Aroclor 1260, when fed in the diet, had a hepatocarcinogenic effect in these rats. No significant differences could be observed between experimental and control animals with regard to the incidence of tumors in other organs. In the study by Kimbrough (*8*), 50 male BALB/cJ mice were fed a diet containing 300 ppm of Aroclor 1254 for a period of 11 months. Another group of 50 male mice received a diet containing 300 ppm of Aroclor 1254 for a period of 6 months and a control rat chow diet for the next 5 months. Control males were fed a plain rat chow diet throughout the 11-month study. Food consumption and body weight were monitored through the study. The animals were sacrificed at the end of 11 months, and the organs were examined grossly for pathology. The liver and any other abnormal-appearing tissues were examined microscopically. Of the 22 surviving animals which had received a diet containing 300 ppm of Aroclor 1254 for 11 months, nine animals exhibited hepatomas. One of the 24 mice which had received a diet containing 300 ppm of Aroclor 1254 for 6 months and the control diet for the next 5 months exhibited a hepatoma. None of the control animals developed hepatomas during the course of the experiment.

Allen and Norback (*22*) have investigated, among other things, PCB-related reproductive disfunctions in the rhesus monkey, an animal species known to be more susceptible than rodents to the toxic effects of PCBs. In one series of experiments, eight female monkeys were fed a diet containing 2.5 ppm of Aroclor 1248 for 6 months, eight other females were fed a diet containing 5.0 ppm of Aroclor 1248, and 12 females served as controls. At the end of 6 months, all experimental and control animals were bred to control males.

Six of the eight animals receiving the test compound at 5.0 ppm in the diet conceived. The remaining two were bred on five separate occasions without conceiving. Four of the six animals which did conceive experienced abortions early in gestation, and one gave birth to a stillborn infant. Eight females receiving diets containing 2.5 ppm of Aroclor 1248 were able to conceive, but only five females in this group were able to carry their infants to term, while the remaining three animals experienced abortions. The infants born to all experimental mothers receiving the test compound at either 2.5

ppm or .0 ppm in the diet were small at birth and exhibit detectable concentrations of PCBs in the skin. A of the 12 control females conceived and had nor al births. Each of the three toxicological studies f PCBs described above was used to assess the car nogenic or reproductive risks posed by these c mpounds.

Risk ssessment

As in icated previously, the scientific data base needed support a quantitative estimate of risk to human alth as a result of exposure to environmental contaminants is nearly always inadequate to quantify such ris accurately. In a typical case, there are at least four major areas of uncertainty (*23*): (1) a lack of adequate information about the exposures that occur in human populations at environmental levels of the contaminant; (2) a lack of dose-response data in humans to support projections of the effects of likely levels of exposure; (3) a comparable lack of dose-response data, even in laboratory animals, at the very low levels of exposure that commonly occur in the environment; and (4) a lack of understanding of the interactions and influences that environmental variables and characteristics of the exposed population may have on the effects of the contaminant.

The state of scientific knowledge at present makes it unlikely that these uncertainties can be eliminated. However, it is possible to simplify the analysis by making a number of assumptions (*23*). One can assume, for instance, that "typical" exposure levels can be calculated from limited data, or can be estimated arbitrarily to encompass what appears to be a reasonable range; that dose-response curves derived from animal studies can be used as analogs to estimate human responses; that the dose-response curve can be extrapolated, using best scientific judgment concerning its probable form, from known responses at high doses to estimated responses at much l ver doses; or that for lack of any sounder choice, he influence of confounding variables can simply e left out of the calculations.

Estir ates of risk based on such an approach have increas gly been attempted: an example is the recent sessment by the National Research Council of halor ethanes in drinking water (*24*). The results, while c ide, have some value in decision-making, if only to ace in perspective the hazards of pollutants whose oxic properties are known qualitatively (e.g., utagenic, carcinogenic). Quantitative assessments of this sort, however, are no stronger than th structure of assumptions on which they rest. Because many of the assumptions are merely pragmatic and are derived from limited information, risk estimates of this type cannot be accepted as conclusive results. They should instead be viewed as initial attempts that are subject to revision as better information becomes available.

In attempting to estimate risks from exposure to PCBs through the consumption of PCB residues in certain species of fish, several problems are readily apparent. One, there is no measurement of PCBs in the fish actually consumed by individuals. PCB residues are measured in fish from one survey, while fish consumption is measured in another. Second, the risks are computed from a variety of animal studies which include different animals species exposed to a variety of Aroclors and levels of Aroclors in the diet with effects measured in different ways, e.g., general toxicity, carcinogenicity and problems of reproduction.

In the risk assessment described below, the average and an upper limit for PCB intake per day are estimated. The nationwide survey of fish consumption conducted for NMFS-NOAA during 1973-1974 has been used to estimate consumption of fish. This survey included 25,947 persons representative of the U.S. population who recorded their fish and seafood consumption for each family member for a one-month period. Of these 25,947 persons, 3939 ate the species of fish which have levels of PCB above 1 ppm.

Since the effect of instituting a tolerance of PCB levels down to 1 ppm would change the PCB contributed to the diet by fish only in those fish which showed PCB levels above 1 ppm, risks corresponding to tolerances of 5 ppm, 2 ppm, 1 ppm, or 0 were calculated for those 3939 persons who ate the species of interest. Because analytical methods for regulatory purposes are not presently available for levels less than 1 ppm, no risks were calculated for lower levels. The calculated risks could then be extrapolated to that proportion of the total U.S. population which is expected to eat these species, that is, 3939/25,947, or 15.2%.

For these persons, the consumption per day of each type of fish was multiplied by a mean PCB level estimated for each tolerance level to give a total PCB intake from fish per person per day. The 50th and 90th percentiles of PCB intake from fish for those eaters of the species of interest were then used to calculate risks.

The mean PCB level estimated when a given tolerance is in effect is perhaps the most difficult part of the risk estimation. The effect of a tolerance on the distribution of PCB levels depends to a large degree on the actual distribution of PCBs before a tolerance is instituted. The most recent data available on PCB levels in fish were the 1978 and 1979 FDA survey data, consisting of 713 samples for 1978 and 179 samples for 1979 collected from all of the FDA districts. This sampling is not representative or

Table 3. Animal data used for risk extrapolation to humans.

Study	Parameter	Dose of Aroclor fed, ppm						
		0	2.5	5.0	25	50	[illegible]	300
Fischer rats fed Aroclor 1254 (*20*)								
	Total malignancies							
Males		5/24			2/24	9/24	1 4	
Females		4/24			13/24	8/24	4	
Combined		9/48			15/48	17/48	2 8	
	Liver carcinoma and adenomas							
Males		0/24			0/24	1/24	4	
Females		0/24			0/24	1/24	4	
Combined		0/48			0/48	2/48	8	
	Hematopoietic system							
Males		3/24			2/24	5/24	24	
Females		4/24			6/24	6/24	24	
Combined		7/48			8/48	11/48	1 48	
Female Sherman rats fed Aroclor 1260 (*21*)	Hepatocellular carcinomas	1/173					2 184	
BALB/cJ male mice fed Aroclor 1254 (*16*)	Hepatomas, neoplastic nodules	0/5						9/22
Female monkeys fed Aroclor 1248 (*22*)[a]	Problems of reproduction	0/12	3/8	7/8				

[a] For the monkeys fed Aroclor 1248, assuming a body weight of 5 kg and daily food consumption of 250 g: 2.5 ppm = 125 μg/kg body weight/day; 5.0 ppm = 250 μ/kg body weight/day.

extensive enough to permit estimation of an underlying nationwide distribution by species. As a rough approximation of the effect of a tolerance on the distribution, the values of PCB above the assumed tolerance were eliminated from the sample distribution and the mean was recalculated for each species. The resulting mean levels are shown in Table 1. It should be noted that assuming a zero tolerance is not equivalent to using all values, inasmuch as the 1975-1979 survey was carried out when a tolerance of 5 ppm was in effect. Thus, the effect of going from zero tolerance to a tolerance of 5 ppm would be greater than shown here. Tuna and shellfish were assumed to have 0.0 mean levels of PCB. The limited data available on tuna show mean levels of less than 0.01 ppm. The values in Table 1 were then multiplied by consumption figures as described above to obtain intake of PCBs per day.

The total intake of PCB per day from fish for eaters at the 50th and 90th percentiles is shown in Table 2. The ppm of the diet is calculated by assuming 1500 g total food intake per person per day.

Data from the NCI bioassay program in which Aroclor 1254 was fed to Fischer rats are presented in Table 3, which shows the numbers for total malignancies, liver carcinomas plus adenomas and malignancies of the hematopoietic system in males, females and males and females combined at various feeding levels. Similar data are also presented in Table 3 for the feeding studies of Kimbrough in which female Sherman rats were fed 100 ppm Aroclor 1260 and BALB female mice were fed 300 ppm Aroclor 1254, and for the Allen reproductive data in which Aroclor 1248 was fed at 2.5 or 5.0 ppm.

Using the data for Table 3, the upper confidence limits (99%) on lifetime risks for cancer and problems of reproduction in eaters of the 12 fish species of interest at the 50th percentile are presented in Table 4. Upper limits on estimated human risks have been computed from the NCI data for total malignancies for males plus females, liver carcinomas plus adenomas in males plus females and ma lignancies of the hematopoietic system in males plu females. Risk computed from the Kimbrough dat and from the Allen data are also presented in Ta e 4. The various risks shown are based on PCB alues in fish, assuming zero tolerance, or a tolera e of 5, 2 or 1 ppm.

In describing the potential risk to hu n health from exposure to PCBs it seems appropr e also to review the general scheme of establi ng safe regulatory levels based on general icity as opposed to carcinogenicity or problem f reproduction. In the Yusho incident, the ividuals consumed an average of 15,000 mg/d of the contaminated oil. The oil itself was conta nated at levels of 2000-3000 ppm PCBs and other co minants (such as polychlorinated quaterphenyls); t average level of the contamination in the oil was 00 ppm. The levels of contamination of the rice il were calculated at the time of the incident by mparing the known organic chlorine content of the rice oil with the known organic chlorine content of anechlor 400.

Based on the two average levels (consumption of rice oil and residue levels in the rice oil), the

Table 4. Upper confidence limits (99%) on lifetime risks of cancer and problems of reproduction in eaters of fish species of interest.

		Lifetime risks per 100,000[a]							
		50th percentile eaters				90th percentile eaters			
Study	Basis parameter/species	Assumed no tolerance	Assumed tolerance = 5 ppm[b]	Assumed tolerance = 2 ppm	Assumed tolerance = 1 ppm	Assumed no tolerance	Assumed tolerance = 5 ppm	Assumed tolerance = 2 ppm	Assumed tolerance = 1 ppm
NCI (*20*)	Total malignancies (male and female rats)	4.1	3.7	2.7	1.6	10.6	9.8	7.2	4.4
NCI (*20*)	Liver carcinoma and adenomas (male and female rats)	0.9	0.9	0.6	0.4	2.5	2.3	1.7	1.0
NCI (*20*)	Hematopoietic (male and female rats)	2.7	2.4	1.8	1.1	7.0	6.5	4.7	2.9
Kimbrou (*21*)	Liver carcinoma	1.3	1.2	0.8	0.5	3.4	3.1	2.3	1.4
Kimbrou (*16*)	Liver hepatomas (mice)	2.0	1.8	1.2	0.8	5.2	4.8	3.5	2.2
Allen (*2*	Female-male reproduction (monkey)[c]	337	307	222	132	883	811	595	367

[a] All risks are liftime risks computed as rates per 100,000 of the population at risk.

[b] For each assumed tolerance, PCB values below the tolerance were eliminated.

[c] Inasmuch as the monkeys were fed Aroclor 1248 for only 6 months, the risk computed for problems of reproduction are not true lifetime risks.

average daily intake of the combination of contaminants was 37.5 mg/day. The average cumulative dose of the contaminants causing an overt effect in the Japanese victims was reported to be 2000 mg. Thus 53 days of exposure was required to consume this amount. The period of exposure no doubt varies around this figure. However, it was estimated that the maximum exposure time was 100 days. It must also be assumed that the adverse health effects result from the combination of contaminants and that these effects are reasonably similar for the levels of PCBs as well as for the levels of chlorinated quaterphenyls or other contaminants.

Humans in the United States have not been exposed to PCBs at the high residue levels that occurred in the Yusho incident. PCB exposure in the United States has been assumed to be sporadic and self-limiting in nature, as far as the general public was concerned. Accordingly, in developing temporary tolerances based on the data from Yusho incident a time period of 1000 days of exposure was used. As previously stated, this was not an analysis based on lifetime exposure. Rather, it was postulated that PCB levels in food in the U.S. would steadily decrease over the 1000-day time period used in the calculation. This has in fact taken place for most food. J inek and Corneliussen (*25*) reported that from the 1969-1975 period, there were significant decreases in PCB levels in all foods with the exception of fish, where no particular trend had been noted.

In calculating a total allowable exposure from the average overt dose in the Yusho incident, a safety factor of 1 to 10 was used for those effects observed in the Yusho population, resulting in a total allowable exposure of 200 mg. Because of the sporadic and self-limiting nature of PCB exposure in the United States, the total exposure (200 mg) was spread out over the 1000-day time period, providing a tolerable daily exposure of no more than 200 μg per day. Transforming this figure and using an average body weight of 70 kg for an adult produced a value of 3 μg/kg body weight/day.

Infants and young children may be more susceptible than adults to toxicants such as PCBs. They also consume a greater amount of food per kilogram of body weight and therefore have a proportionately greater exposure to PCBs than adults. Thus, in calculating the temporary tolerances, it seems appropriate to use an additional safety factor for infants and young children. The acceptable daily exposure for children is therefore calculated by using the lowest total dose producing an adverse health effect in the Yusho incident, which was determined to be 500 mg of the contaminants. Using the 1:10 safety factor spread over 1000 days, the tolerable daily exposure is 50 μg/day. Infants and young children should, therefore, not be exposed to PCBs at a level greater than 1 μg/kg body weight/day. An adult who consumes a balanced and varied diet should not be expected to ingest more than the tolerable daily exposure of 200 μg/day. Similarly, infants or young children consuming a balanced and varied diet should not be expected to ingest more than their tolerable daily exposure.

In addition to the human epidemiological data, other data from long-term animal studies (two years) had appeared to establish that the no-effect level in rats and dogs for PCBs with three levels of chlorination (42, 54 or 60%) was 10 ppm. These animal data have been used with the epidemiological data to estimate allowable daily intake in humans. When data derived from dogs were used, a no-effect level of 2.5 μg/kg body weight/day was estimated. When rat data were used, the estimated no-effect level in man was 3 μg/kg body weight/day or a level similar to the human epidemiological data. Thus, for a 70 kg individual, an allowable level of PCB ingestion would be 175 to 210 μg/day. However, more recent analysis of these data has raised serious questions as to the validity of the original interpretation of results. These data appear to offer little help in arriving at an allowable daily intake level of PCBs in humans.

Discussion

There is considerable disparity in reproductive results of monkeys compared to other species. Yet the consuming public tends to consider that monkeys are more like humans than rodents are. Thus it is necessary to consider why such disparities exist. It has been pointed out by McNulty (*26*) and by Allen et al. (*22*, *27*) that rhesus monkeys are very sensitive to PCBs, not only in the subacute effects, but in problems of reproduction as well. These monkeys received 2.5 ppm or 5.0 ppm in the diet; over a period of 6 months, total intake ranged from 250 to 400 mg.

In contrast, Keplinger et al. (*28*) reported low mating indices and decreased survival of pups in rodents receiving Aroclor 1242 at 100 ppm. No reproduction effects were found with Aroclor 1242 or 1254 at 1 or 10 ppm although other studies in rodents (*29*) have demonstrated reproduction effects; e.g., in Sherman rats exposed to Aroclor 1254 or Aroclor 1260, the exposures producing such effects have been at levels considerably higher than the 2.5 or 5.0 ppm used in the monkey studies (20, 100 or 500 ppm).

Additional differences between the monkey and rodent sensitivities to PCBs are illustrated by the fact that the Sherman rats in the Kimbrough study fed 100 ppm of Aroclor 1260 for 23 months apparently exhibited no subacute effects, which is also true of the NCI study in which Fischer rats were fed 25, 50 or 100 ppm for 104-105 weeks. In contrast, the monkeys fed 2.5 or 5.0 ppm in the Allen study displayed some subacute effects similar to Yusho as early as 2 months into the study.

The data upon which the various risk projections of carcinogenicity are made also illustrate the difficulties in such exercises. For example, the data used for projection of risk for Aroclor 1254, the most common Aroclor of human exposure, are taken from an NCI bioassay project in which the Aroclor 1254 was found under the conditions of the test to be negative for carcinogenicity. In addition, an examination of Table 4 shows that the upper limit of lifetime risk of cancer for the 50th percentile of eaters of the species of interest is generally of the same magnitude in any of the studies shown.

Let us assume that what appear to be increased risks in reproduction from exposure to PCBs based on monkey studies are directly associated with the levels fed, the particular Aroclor fed and the differences in species sensitivity, i.e., the monkeys are considerably more sensitive to the effect of PCBs than are rodents or humans. It then becomes difficult to explain the differences between the Kimbrough study, which was positive for carcinogenicity in female Sherman rats fed Aroclor 1260 at 100 ppm, and the NCI feeding studies which were negative for Aroclor 1254 fed to Fischer rats at 25, 50 or 100 ppm.

These differences in carcinogenic outcome could be attributed to any of several causes: (a) the Kimbrough study used Sherman rats whereas the NCI study used Fischer rats; (b) three levels of the Aroclor were fed in the NCI study but only one in the Kimbrough study; (c) Kimbrough used 184 test animals at the 100 ppm level, whereas NCI used only 24 animals in each sex group at the 100 ppm level. On the other hand, the difference may be purely statistical in which outcome could be changed in either direction by using comparable protocols and a similar number of animals. Obviously the other elements of unknown quantity in these or any other similar assessments involve the adequacy of the fish consumption data as well as the PCB residue data.

Summary

In any estimates of human risk derived from the extrapolation of animal data, close attention should be given not only to the levels of exposure to the various Aroclors in a variety of animal studies but also to the way in which the exposure relates to human experience. For example, studies in monkeys have reported signs and symptoms similar to those of Yusho after 2 months of exposure to Aroclor 1248 at levels of 2.5 and 5.0 ppm in the diet or 125 and 250 μg/kg body weight/day, respectively. Reproduction problems were reported in these monkey studies at each of these levels after 6 months of exposure.

In contrast, problems of reproduction have been observed in rodents only at considerably higher levels of exposure, e.g., in rats fed 7.2 and 37.0 mg/kg body weight/day of Aroclor 1254 or 100 and 500 ppm in the diet. Rats fed 500 ppm or 35.4 mg/kg body eight/day of Aroclor 1260 also exhibited reprod ction problems. No problems were observed with A oclor 1260 at levels of 5 ppm (0.39 mg/kg body w ight/day), 20 ppm (1.5 mg/kg body weight/day) o 100 ppm (7.4 mg/kg body weight/day).

In o study, the carcinogenicity of PCBs in rats (Fisch strain) fed Aroclor 1254 at 25 ppm (1.9 mg/kg ody weight/day), 50 ppm (3.8 mg/kg body weight ay) or 100 ppm (7.16 mg/kg body weight/day) was re rted to be negative under the test conditions. Althou h some malignancies were observed, there was n statistical difference between test animals and co trols. In another study, female Sherman rats fe Aroclor 1260 at 100 ppm (7.4 mg/kg body weight day) exhibited a statistically significant difference between test animals and controls for hepato ellular carcinomas.

In contrast, there appears to be little evidence of human exposure to these levels in the United States, especially for the consumption of fish. Even in the Yusho experience in Japan, where clinical signs and symptoms were observed, the average level of consumption of PCB residues in the rice oil is estimated to have been 0.75 mg/kg body weight/day.

In the United States, estimates of the daily intake of PCBs for eaters in the 50th percentile are 8.46 μg/day or 0.72 μg/kg body weight/day (based on a 70 kg individual) assuming no PCB tolerance; 7.57 μg/day or 0.11 μg/kg body weight/day assuming a tolerance of 5 ppm, 5.59 μg/day or 0.08 μg/kg body weight/day assuming a tolerance of 2 ppm and 3.30 μg/day or 0.05 μg/kg body weight/day assuming a tolerance of 1 ppm. Estimates of the intake of PCBs for eaters in the 90th percentile are 0.32 μg/kg body weight/day assuming no tolerance and 0.29, 0.21 and 0.13 μg/kg body weight/day assuming a toler nce of 5, 2, and 1 ppm, respectively.

In M chigan sportsfishermen, who are presumed to be a nong the high consumers of fish with PCB residu , the average intake has been reported at 1.7 μg g body weight/day with a range of 0.09 to 3.94 μ kg body weight/day.

Prob ems of interpretations arise in comparing the le els of the various Aroclors which have produc d effects in animals, ranging from 125 μg in monke s to the milligram levels in rodents, with the ex osure estimates in humans from fish consumpt n. For example, estimates of the lifetime human risk of cancer and reproduction problems (Table) for exposure in the 90th percentile of fish eaters, i.e., 0.29, 0.21 and 0.13 μg PCB/kg body weight day, indicate risk from exposure well below the av rage Michigan exposure and certainly well below he levels of exposure in the Yusho incident.

In li ht of the uncertainties upon which these risk es imates have been made, perhaps an equally compelling argument could be made for the establishment of either a 2 ppm or a 1 ppm tolerance. As suggested previously, the difference in risk between the two levels decreases only slightly even in the species of interest. From one point of view, this conclusion supports a rationale for proposing the 2 ppm tolerance based on the original calculation of an allowable daily intake resulting from the traditional use of the 1 to 10 safety factor which has been described in the previous section. In this case some similarities exist even in the face of uncertainty. To some, the rationale and logic for establishing 2 ppm can be justified from either the risk approach or the so-called safety approach. To others, nothing short of zero tolerance has any rationale or logic. For these, there may be no answer.

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National Toxicology Program

Environmental Health Perspectives
Vol. 45, pp. 185-198, 1982

Carcinogenesis Bioassay Results from the National Toxicology Program

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This article begins a series of condensations on the National Toxicology Program's (NTP) toxicology and carcinogenesis bioassay results as summarized from the *NTP Technical Report* collection. This first compilation lists those 40 carcinogenesis bioassay studies (Table 1) that have undergone both NTP staff interpretation and evaluation as well as external peer review and critique. The majority of these studies were initiated by the National Cancer Institute's Carcinogenesis Testing Program, now part of the National Institute of Environmental Health Sciences, National Toxicology Program.

Précis on individual *NTP Technical Reports* will be submitted for publication in *Environmental Health Perspectives* (*EHP*) at the same time that the approved reports are delivered for printing. For instance, information and data condensations on four recently printed carcinogenesis bioassay reports [caprolactam, cytembena, di(2-ethylhexyl) adipate, and FD&C Yellow No. 6] appear as separate inserts following this introductory article. Considerably more abbreviated results are published routinely in the *NTP Technical Bulletins*. More extensively treated manuscripts are being prepared for publication consideration in *EHP* and in other recognized peer-review journals. This allows not only more rapid and wider dissemination of results generated by the NTP, but permits open interchange between scientists/organizations engaged or interested in similar research and testing activities.

For orientation purposes, the following overview highlights the National Toxicology Program, the chemical nomination and selection process, and the toxicology and carcinogenesis bioassay program.

National Toxicology Program

The NTP completed its fourth year of operation and first year in permanent status in Fiscal Year (FY) 1982. The NTP was established in November 1978 as a Department of Health and Human Services (DHHS) cooperative effort to coordinate and provide information about potentially toxic chemicals to regulatory and research agencies and to strengthen the science base in toxicology. The need for creation of such a program evolved from increasing scientific, regulatory, and congressional concerns about the human health effects of chemical agents in the environment.

A major reorganization occurred within NIH in FY 1981: the transfer of the carcinogenesis bioassay program from the National Cancer Institute (NCI) to the National Institute of Environmental Health Sciences (NIEHS) was approved by the Secretary, DHHS, in the fourth quarter of FY 1981. Further, at the beginning of FY 1982, the Secretary granted permanent status to the National Toxicology Program.

Under the broad objective of providing the necessary scientific information for prevention of human disease related to chemical exposure, the NTP emphasizes four principal goals: (1) broaden the toxicological characterization of chemicals that are tested; (2) increase the rate of chemical testing (as funding will permit); (3) develop protocols appropriate for regulatory needs; and (4) communicate program plans and results to governmental agencies, the medical and scientific communities, and the public.

Although testing chemicals for toxicity, particularly for carcinogenicity and mutagenicity, remains an important program focus, increasing emphasis over the first four years has been placed on short-term test methods development and validation. A second primary emphasis continues to be

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Table 1. National Toxicology Program toxicology and carcinogenesis bioassay results.

Chemical CAS No. NTP No.	NTP Report No. TR Report No. Peer Review Date	Use	Route/dose	Testing laboratory	NTP chemical manager	Results under the conditions of these tests[b,c]
Agar 9002-18-0 10754-C	NTP-80-084 TR-230 P[a] 02/18/81	Gelling agent in foods and pharmaceuticals	Feed: 25,000 or 50,000 ppm	EG&G Mason Research Institute	Dr. R. L. Melnick	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
Allyl isothiocyanate 57-06-7 10367-A	NTP-81-036 TR-234 06/23/81	Flavor agent, major component in brown mustard seed	Gavage (corn oil): 12 or 25 mg/kg 5 times/week	Southern Research Institute	Dr. J. Dunnick	Carcinogenic to male F344 rats causing transitional cell papilloma in the urinary bladder (0/49, 2/49, 4/49). (Additionally, epithelial hyperpalsia of the urinary bladder occurred at 0/49, 1/49, 6/49 in male rats not having papillomas.) Not carcinogenic to female F344 rats or B6C3F$_1$ mice of either sex.
11-Aminoundecanoic acid 2432-99-7 10783-W	NTP-80-034 TR-216 P 02/18/81	Monomer used in manufacture of polyamide nylon 11	Feed: 7500 or 15,000 ppm	Litton Bionetics Inc.	Dr. J. Dunnick	Carcinogenic for male F344 rats, inducing neoplastic nodules of the liver (1/50, 9/50*, 8/50*) and transitional-cell carcinomas in the urinary bladder (0/48, 0/48, 7/49*); not carcinogenic for female F344 rats. Not carcinogenic for B6C3F$_1$ mice of either sex, although the increase in male mice with malignant lymphoma (2/50, 9/50*, 4/50) may have been associated with the administration of 11-aminoundecanoic acid.
Arabic gum 9000-01-5 10751-P	NTP-80-081 TR-227 P 02/18/81	GRAS food additive, flavor fixative, foam stabilizer, emulsifier	Feed: 25,000 or 50,000 ppm	EG&G Mason Research Institute	Dr. J. E. Huff	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
Asbestos, amosite 12172-73-5 10761-X	NTP-81-058 TR-249 06/23/81	Asbestos-cement pipe	1% in diet	Illinois Inst. of Technology Research Inst.	Dr. E. E. McConnell	Not carcinogenic for Syrian golden hamsters of either sex.
Asbestos, chrysotile 12001-29-5 10758-S	NTP-81-051 TR-246 06/23/81	Asbestos-cement, textiles, insulation	1% in diet (short-range, SR; intermediate range, IR)	Illinois Inst. of Technology Research Inst.	Dr. E. E. McConnell	Not carcinogenic for Syrian golden hamsters of either sex. [Increased incidence of adrenal cortical adenomas in hamsters exposed to IR chrysotile when compared to pooled controls—males: 31/466 (7%) versus 29/244 (12%)*; females: 19/468 (4%) versus 23/234 (10%)*].
Asbestos, [illegible] 1,2-dimethylhydrazine (CAS no. 540-73-8) 12001-29-5 10758-S	NTP-81-051 06/23/81	Asbestos- textiles, insulation; positive control, intestinal carcinogen	1% in diet, (intermediate range, IR); gavage (4 mg/kg every other week for 5 doses)	Illinois Inst. of Technology Research Inst.	Dr. E. E. McConnell	Combination study in Syrian golden hamsters of either sex [illegible] no increase in DMH-induced intestinal neoplasia (DHM is known to induce gastrointestinal tumors in animals).

2-Biphenylamine hydrochloride (2-aminobiphenyl) 2185-92-4 10438-K	NTP-81-035 TR-233 06/23/81	Chemical intermediate for C.I. Acid Red 15	Diet: 1000 and 3000 ppm	EG&G Mason Research Institute	Dr. K. M. Abdo	Not carcinogenic for F344 rats of either sex. Equivocal for male B6C3F$_1$ mice causing hemangiosarcoma of the circulatory system 0/50, 2/50, 3/50 (however, [illegible]) for female B6C3F$_1$ mice causing hemangiosarcomas of the circulatory system (0/49, 1/50, 7/50*).
Bis(2-chloro-1-methyl ethyl) ether (BCMEE) 108-60-1 10523-N	NTP-81-055 TR-239 12/16/81	Paint and varnish removers; intermediate in dyes, resins and pharmaceuticals manufacture; and nematocide in Japan	Gavage (corn oil) 5 times/week: mice, 100 or 200 mg/kg	EG&G Mason Research Institute	Dr. M. Powers	Carcinogenic for B6C3F$_1$ mice causing alveolar/bronchiolar adenomas in males (5/50, 13/50*, 11/50) and in females (1/50, 4/50, 8/50*) and inducing in males dose-related hepatocellular carcinomas (5/50, 13/50, 17/50*). Rare forestomach tumors were probably associated with the test chemical (low dose: 1 male; high dose: 2 males and 3 females).
Bisphenol A 80-05-7 10034-Y	NTP-80-035 TR-215 P 10/15/80	Intermediate in manufacture of resins, flame retardants, and rubber chemicals	Feed: rats, 1000 or 2000 ppm; mice, 1000 or 5000 ppm 5000 or 10,000 ppm (female)	Litton Bionetics, Inc.	Dr. J. E. Huff	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex (increased incidences of leukemia in male rats 13/50, 12/50, 23/50* and lymphoma in male mice 2/49, 8/50*, 3/50 may have been associated with the test chemical).
Butyl benzyl phthalate 85-68-7 10422-E	NTP-80-025 TR-213 06/27/80	Additive to polymers (PVC) for flexibility and softness; plasticizer	Feed: 6000 or 12,000 ppm	EG&G Mason Research Institute	Dr. J. E. Huff	Male F344 rat data inadequate for evaluation (early death caused by hemorrhage); probably carcinogenic for female F344 rats, causing an increased incidence of mononuclear cell leukemia (7/49, 7/49, 18/50*); not carcinogenic for B6C3F$_1$ mice of either sex.
Caprolactam 105-60-2 10505-F	NTP-80-026 TR-214 P 06/27/80	To produce polycaprolactam (nylon 6); manufacture of polyamide synthetic fibers	Feed: rats, 3750 or 7500 ppm; mice, 7500 or 15,000 ppm	Litton Bionetics, Inc.	Dr. J. Dunnick	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
C.I. Acid Orange 10 1936-15-8 10693-Y	NTP-80-030 TR-211 02/18/81	Monoazo dye, in biological materials; paper, wood, leather, wool, silk, inks; and pencil coatings	Feed: rats, 1000 or 3000 ppm; mice, 3000 or 6000 ppm	Battelle Columbus Laboratories	Dr. J. E. Huff	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
C.I. Acid Red 14 3567-69-9 10723-Y	NTP-80-067 TR-220 P 10/15/80	Dye for nylon, silk, wool, leather, paper, wood	Feed: rats, 6000 or 12,000 ppm (male) and 12,000 or 25,000 ppm (female); mice 3000 or 6000 ppm	Battelle Columbus Laboratories	Dr. R. D. Irwin	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.

Table 1. *(continued)*

Chemical CAS No. NTP No.	NTP Report No. TR Report No. Peer Review Date	Use	Route/dose	Testing laboratory	NTP chemical manager	Results under the conditions of these tests[b,c]
C.I. Disperse Yellow 3 2832-40-8 10716-D	NTP-81-080 TR-222 P 02/18/81	Monoazo dye in nylon, wool, fur, poly(vinyl chloride), acrylic fibers, polystyrene, other thermoplastics	Feed: rats 5000 or 10,000 ppm; mice, 2500 or 5000 ppm	Battelle Columbus Laboratories	Dr. J. H. Mennear	Carcinogenic for male F344 rats, causing neoplastic nodules of the liver (1/49, 15/50*, 10/50*); rare stomach tumors may have been associated with the test chemical (0/49, 4/50, 1/50). Not carcinogenic for female rats or male B6C3F$_1$ mice. Carcinogenic for female mice causing hepatocellular adenomas (0/50, 6/50*, 12/50*).
C.I. Solvent Yellow 14 842-07-9 10662-W	NTP-80-081 TR-226 02/18/81	Monoazo dye in hydrocarbon solvents, oils, fats, shoe polish, gasoline, soap	Feed: rats, 250 or 500 ppm; mice, 500 or 1000 ppm	Battelle Columbus Laboratories	Dr. R. D. Irwin	Carcinogenic for F344 rats, inducing neoplastic nodules of the liver in both males (5/50, 10/50, 30/50*) and females (2/50, 3/49, 10/48*); not carcinogenic for B6C3F$_1$ mice of either sex.
Cytembena 21739-91-3 10778-H	NTP-80-027 TR-207 P 06/27/80	Cytostatic agent	IP, 3 times/week: rats, 7 or 14 mg/kg; mice, 12 or 24 mg/kg	Southern Research Institute	Dr. C. Grieshaber	Carcinogenic for F344 rats (male: mesotheliomas in the tunica vaginalis 0/50, 11/50*, 10/50* and in multiple organs 3/50, 26/50*, 26/50*; female: fibroadenomas in the mammary gland 13/49, 22/50, 36/50*); not carcinogenic for B6C3F$_1$ mice of either sex.
D&C Red No. 9 5160-02-1 10729-X	NTP-80-079 TR-225 P 02/18/81	Pigment in topical drugs and cosmetics	Feed: rats, 1000 or 3000 ppm; mice, 1000 or 2000 ppm	Battelle Columbus Laboratories	Dr. C. Grieshaber	Carcinogenic in male F344 rats inducing sarcomas of the spleen (0/50, 0/50, 26/48*) and neoplastic nodules in the liver (0/50, 6/50*, 7/49*); not carcinogenic for female F344 rats, although the increased number of female rats with neoplastic nodules of the liver (1/50, 1/50, 5/50) may have been related to compound administration. not carcinogenic for B6C3F$_1$ mice of either sex.
1,2-Dibromo-3-chloropropane (DBCP) 96-12-8 10465-X	NTP-81-021 TR-206 P 06/27/80	Soil fumigant for the control of nematodes	Inhalation, 6 hr/day, 5 days/week: 0.6 or 3.0 ppm	Hazleton Laboratories America, Inc.	Dr. J. E. Huff	Carcinogenic for F344 rats (nasal cavity tumors – male: 0/50, 40/50*, 39/49*; female: 1/50, 27/50*, 42/50* and tumors of tongue – male: 0/50, 1/50, 11/49*; female: 0/50, 4/50, 9/50* and cortical adenomas of the adrenal glands in females 0/50, 7/50*, 5/48*). Carcinogenic for B6C3F$_1$ mice (nasal cavity tumors – male: 0/45, 1/42, 21/48*; female: 0/50, 11/50*, 38/50* and alveolar/bronchiolar adenomas – male: [illegible] 3/49, 10/47*).

1,2-Dibromoethane (ethylene dibromide) [illegible] 10513-F	NTP-80-028 TR-210 P 06/27/80	Scavenger for lead in gasoline, soil and grain fumigant, intermediate in dye and drug syntheses	Inhalation, 6 hr/day, 5 days/week: 10 or 40 ppm	Hazleton Laboratories America, Inc.	Dr. M. Powers	Carcinogenic for F344 rats (both sexes – tumors of the nasal cavity and hemangiosarcomas of the circulatory system; male – mesotheliomas in the tunica vaginalis, [illegible] tumors and mammary fibroadenomas). Carcinogenic for B6C3F$_1$ mice (both sexes – alveolar/bronchiolar adenomas and carcinomas; female – hemangiosarcomas, subcutaneous fibrosarcomas, nasal cavity tumors, and mammary gland adenocarcinomas).
2,6-Dichloro-*p*-phenylenediamine 60[illegible] 20-1 10647-A	NTP-80-036 TR-219 P 10/15/80	Intermediate in manufacture of polyamide fiber; a polyurethane curative	Feed: rats, 1000 or 2000 ppm (males) and 2000 or 6000 ppm (females); mice, 1000 or 3000 ppm	Litton Bionetics, Inc.	Dr. C. Whitmire	Not carcinogenic for F344 rats. Carcinogenic for B6C3F$_1$ mice (male: hepatocellular adenomas 4/50, 7/50, 15/50*, carcinomas not significant 12/50, 13/50, 17/50; female: hepatocellular carcinomas 2/50, 2/50, 7/50 and adenomas 4/50, 4/50, 9/50 combined 6/50, 6/50, 16/50*).
Di(2-ethylhexyl) adipate 103-23-1 10497-E	NTP-80-029 TR-212 P 06/27/80	Plasticizer in vinyl plastics	Feed: 12,000 or 25,000 ppm	EG&G Mason Research Institute	Dr. M. Powers	Not carcinogenic for F344 rats of either sex. Carcinogenic for B6C3F$_1$ mice (male: hepatocellular adenomas 6/50, 8/49, 15/49*; female: hepatocellular carcinomas 1/50, 14/50*, 12/49*).
Di(2-ethylhexyl) phthalate (DEHP) 117-81-7 10188-J	NTP-80-037 TR-217 P 10/15/80	Plasticizer for PVC polymers	Feed: rats, 6000 or 12,000 ppm; mice, 3000 or 6000 ppm	EG&G Mason Research Institute	Dr. J. Douglas	Carcinogenic for F344 rats (male: hepatocellular carcinomas and neoplastic nodules combined 3/50, 6/49, 12/49*; female: hepatocellular carcinoma 0/50, 2/49, 8/50*). Carcinogenic for B6C3F$_1$ mice (hepatocellular carcinomas—male: 9/50, 14/48, 19/50*; female: 0/50, 7/50*, 17/50*).
Eugenol 97-53-0 10468-J	NTP-80-068 TR-223 02/18/81	Flavoring agent and fragrance, GRAS food additive	Feed: female rats, 6000 or 12,500 ppm; male rats and mice, 3000 or 6000 ppm	Southern Research Institute	Dr. J. A. Moore	Not carcinogenic for F344 rats of either sex; equivocal for B6C3F$_1$ mice (male: hepatocellular adenomas 4/50, 13/50*, 10/49 or carcinomas 10/50, 20/50*, 9/49 combined 14/50, 28/50*, 18/49; female: hepatocellular carcinomas 2/50, 3/49, 6/49 or adenomas 0/50, 4/49, 3/49 combined 2/50, 7/49, 9/49*).
FD&C Yellow No. 6 2783-94-0 10714-V	NTP-80-033 TR-208 P 06/27/80	Food and cosmetics coloring agent	Feed: 12,500 or 25,000 ppm	Battelle Columbus Laboratory	Dr. M. P. Dieter	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
Guar gum 9000-30-0 10752-T	NTP-80-083 TR-229 P 02/18/81	GRAS food additive, cosmetic stabilizer, pharmaceutical tablet binder	Feed: 25,000 or 50,000 ppm	EG&G Mason Research Institute	Dr. M. P. Dieter	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.

Table 1. *(continued)*

Chemical CAS No. NTP No.	NTP Report No. TR Report No.[a] Peer Review Date	Use	Route/dose	Testing laboratory	NTP chemical manager	Results under the conditions of these tests[b,c]
Locust bean gum (carob seed gum) 9000-40-2 10753-X	NTP-80-066 TR-221 P 10/15/80	Stabilizer, thickener, and binder in foods and cosmetics	Feed: 25,000 or 50,000 ppm	EG&G Mason Research Institute	Dr. J. E. Huff	Not carcinogenic for F344 rats or $B6C3F_1$ mice of either sex.
D-Mannitol 69-65-8 10368-L	NTP-81-052 TR-236 12/16/81	Chewable tablets, sugar replacement, candies and gums, osmotic diuretic, and fluid replacement	Feed: 25,000 or 50,000 ppm	Southern Research Institute	Dr. K. M. Abdo	Not carcinogenic for F344 rats or $B6C3F_1$ mice of either sex.
Pentachloroethane 76-01-7 10149-G	NTP-81-034 TR-232 06/23/81	Solvent; intermediate (formerly) for tetrachloroethane manufacture	Gavage (corn oil): rats, 75 and 150 mg/kg, 5 times/week; mice, 250 and 500 mg/kg, 5 times/week	Gulf South Research Institute	Dr. J. H. Mennear	Not carcinogenic for F344 rats of either sex (nephrotoxic for males: diffuse inflammation – 4/50, 14/49*, 33/50*; mineralization – 4/50, 29/49*, 29/50*); carcinogenic for $B6C3F_1$ mice (male: hepatocellular carcinoma 4/48, 26/44*, 7/45; female: hepatocellular carcinoma 1/46, 28/42*, 13/45* and hepatocellular adenoma 2/46, 8/42*, 19/45*).
Polybrominated biphenyl mixture (Firemaster FF-1) 67774-32-7 10800-C	NTP-81-032 TR-244 06/23/81	Flame retardant	Gavage (corn oil): 0, 0.1, 0.3, 1.0, 3.0, and 10 mg/kg, 5 times/week for 6 months, held for lifetime observation	NIEHS/NTP	Dr. B. N. Gupta	Carcinogenic for F344 rats (male: hepatocellular carcinoma 0/33, 2/39, 0/40, 1/33, 7/33*, 7/31*; female: neoplastic nodules 0/20, 2/21, 0/21, 2/11, 5/19*, 8/20* and hepatocellular carcinoma 0/20, 0/21, 0/21, 0/11, 3/19, 7/20*); carcinogenic for $B6C3F_1$ mice causing hepatocellular carcinoma (male: 12/25, 8/27, 8/24, 12/25, 15/23, 21/22*; female: 0/13, 0/19, 2/15, 2/11, 3/17, 7/8*).
Propyl gallate 121-79-9 10564-Y	NTP-81-042 TR-240 12/16/81	Antioxidant in foods containing fats/oils, in food packaging and in cosmetics	Feed: 6000 or 12,000 ppm	Southern Research Institute	Dr. K. M. Abdo	Not considered carcinogenic for F344 rats, although there was evidence of an increased number of male rats with preputial gland tumors (1/50, 8/50*, 0/50), islet-cell adenomas of the pancreas (0/50, 8/50*, 2/50), and pheochromocytomas of the adrenal glands (4/50, 13/48*, 8/50); rare tumors of the brain occurred in female rats (0/50, 2/50, 0/49). Not carcinogenic for $B6C3F_1$ [illegible] increased number of male mice with malignant lymphoma (1/50, 3/49, 8/50*) may have been associated with the administration of propyl gallate.

Stannous chloride 7772-99-8 [illegible]	NTP-81-033 TR-231 06/23/81	Food preservative, reducing agent in tinplating, stabilizer (colors, perfumes, soaps)	Diet: 1000 or 2000 ppm	Southern Research Institute	Dr. M. P. Dieter	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex. (Increase in C-cell tumors of the thyroid gland in male rats may have been [illegible] chemical: adenomas 2/50, 9/49*, 5/50 or carcinomas 0/50, 4/49, 3/50 combined 2/50, 13/49*, 8/50*.)
Tara gum 39300-88-4 10793-E	NTP-80-078 TR-224 P 02/18/81	Formerly cosmetic stabilizer, no longer imported into U.S.	Feed: 25,000 or 50,000 ppm	EG&G Mason Research Institute	Dr. C. Grieshaber	Not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex.
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) 1746-01-6 10157-G	NTP-80-031 TR-209 P 06/27/80	Chemical contaminant in trichlorophenols; 2,4,5-trichlorophenoxyacetic acid; hexachlorophene	Gavage, 2 days/week: rats and male mice-0.01, 0.05, or 0.5 mg/kg/wk; female mice-0.04, 0.2, or 2.0 mg/kg/wk	Illinois Inst. of Technology Research Inst.	Dr. J. A. Moore	Carcinogenic for Osborne-Mendel rats (male: follicular cell thyroid adenomas 1/69, 5/48*, 6/50*, 10/50*; female: neoplastic nodules of the liver 5/75, 1/49, 3/50, 12/49*); carcinogenic for B6C3F$_1$ mice (male: hepatocellular carcinomas 8/73, 9/49, 8/49, 17/50*; female: 1/73, 2/50, 2/48, 6/47*; female: follicular cell adenoma 0/69, 3/50, 1/47, 5/46*).
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) 1746-01-6 10157-G	NTP-80-032 TR-201 P 06/27/80	Chemical contaminant in trichlorophenols; 2,4,5-trichlorophenoxyacetic acid; hexachlorophene	Dermal, 3 days/week: male mice, 0.001 mg; female mice, 0.005 mg	Illinois Inst. of Technology Research Inst.	Dr. J. A. Moore	Not carcinogenic for male Swiss-Webster mice; (increase in fibrosarcomas of the integumentary system—3/42, 7% versus 6/28, 21%—may have been associated with skin application of TCDD); carcinogenic for female Swiss-Webster mice (fibrosarcomas of the integumentary system—2/41, 5% versus 8/27*, 30%).
2,3,7,8-TCDD and dimethylbenzanthracene (DMBA) 1746-01-6 10157-G	NTP-80-032 TR-201 P 06/27/80	Chemical contaminant in trichlorophenols; 2,4,5-trichlorophenoxyacetic acid; hexachlorophene	DMBA—50 mg 1 week prior to TCDD dermal	Illinois Inst. of Technology Research Inst.	Dr. J. A. Moore	Initiation-promotion study considered inadequate because DMBA was not tested alone and DMBA-TCDD induced fibrosarcoma incidence not greater than that observed with TCDD alone (Swiss-Webster mice).
1,1,1,2-Tetrachloroethane 630-20-6 10651-J	NTP-81-053 TR-237 12/16/81	Chemical intermediate for producing tri- and tetrachloroethylene	Gavage (corn oil) 5 times/week: rats, 125 or 250 mg/kg; mice, 250 or 500 mg/kg	Gulf South Research Institute	Dr. L. Birnbaum	Not demonstrated carcinogenic for F344 rats (increased incidence of combined neoplastic nodules and hepatocellular carcinomas in males 0/49, 1/49, 3/48 and fibroadenomas of mammary gland in females 6/49, 15/49*, 7/46 may have been associated with test chemical). Carcinogenic in B6C3F$_1$ mice (hepatocellular carcinomas - females: 1/49, 5/46, 6/48*; hepatocellular adenomas - male: 6/48, 14/46*, 21/50*; females 4/49, 8/46, 24/48*).

Table 1. *(continued)*

Chemical CAS No. NTP No.	NTP Report No. TR Report No. Peer Review Date	Use	Route/dose	Testing laboratory	NTP chemical manager	Results under the conditions of these tests[b,c]
Vinylidene chloride (1,1-dichloroethylene) 75-35-4 10109-A	NTP-80-082 TR-228 02/18/81	Intermediate for 1,1,1-trichloroethane, monomer for vinylidene copolymers	Gavage, 5 times/week: rats, 1 or 5 mg/kg; mice, 2 or 10 mg/kg	Gulf South Research Institute	Dr. R. S. Chhabra	Not carcinogenic for F344 rats or $B6C3F_1$ mice of either sex (increased incidence of liver necrosis in male mice 1/46, 3/46, 7/49*).
Zearalenone 17924-92-4 10770-C	NTP-81-054 TR-235 12/16/81	Nonsteroid estrogenic mycotoxin (anabolic steroid)	Feed: rats, 25 or 50 ppm; mice, 50 or 100 ppm	Southern Research Institute	Dr. D. Goldman	Not carcinogenic for F344 rats of either sex; should be considered carcinogenic for $B6C3F_1$ mice (male: pituitary adenoma 0/40, 4/45, 6/44*; female: pituitary adenoma 3/46, 2/43, 13/42*; hepatocellular adenoma 0/50, 2/49, 7/49*).
Ziram (Zinc dimethyldithiocarbamate) 137-3-4 10590-G	NTP-81-057 TR-238 12/16/81	Fungicide and accelerator in rubber vulcanization	Feed: rats, 300 or 600 ppm; mice, 600 or 1200 ppm	Southern Research Institute	Dr. D. Goldman	Carcinogenic for male F344 rats causing C-cell carcinomas of the thyroid 0/50, 2/49, 7/49*; not carcinogenic for female F344 rats or for male $B6C3F_1$ mice; interpretation of the increased number of female $B6C3F_1$ mice with alveolar/bronchiolar adenoma (2/50, 5/49, 10/50*) is complicated because of a concomitant Sendai viral infection (in all groups of mice).

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[a] P = published.

[b] Incidence results are ordered by concurrent controls, low dose, and high dose groups, with the numerator being the number of tumor (or noneoplastic lesion) bearing animals and the denominator the number of animals examined.

[c] * = statistically significant, $p < 0.05$.

the broadening of the testing protocols through addition of other specific studies to the prechronic phase (single, 14-day, and 90- or 120-day studies) of the long-term bioassay and, to a lesser extent, to the chronic phase (104 weeks) of the bioassay.

Accomplishments of NTP programs during FY 1981 and program plans for FY 1982 are described in the *NTP Annual Plan for Fiscal Year 1982.*

NTP Chemical Nomination and Selection

Because more chemicals are nominated for NTP consideration than can be selected for study, the NTP Executive Committee formulated a set of program guidelines. These resultant eight chemical selection criteria motivate an NTP matrix which operates throughout the NTP. All research, testing and test development/validation efforts start here.

The NTP Executive Committee acts under the principle that industry will test chemicals for health and environmental effects as intended and mandated by Congress under legislative authorities. Therefore, the NTP, using these chemical selection principles, will test: (1) chemicals found in the environment that are not closely associated with commercial activities; (2) desirable substitutes for existing chemicals, particularly therapeutic agents, that might not be developed or tested without federal involvement; (3) chemicals that should be tested to improve scientific understanding of structure-activity relationships and thereby assist in defining groups of commercial chemicals that should be tested by industry; (4) certain chemicals tested by industry, or by others, the additional testing of which by the federal government is justified to verify the results; (5) previously tested chemicals for which other testing is desirable to cross-compare testing methods; (6) "old chemicals" with the potential for significant human exposure which are of social importance but which generate too little revenue to support an adequate testing program (some of these may be "grandfathered" under FDA laws); (7) two or more chemicals together, when combined human exposure occurs (such testing probably cannot be required of industry if the products of different companies are involved); and (8) in special situations, as determined by the Executive Committee, marketed chemicals which have potential for large-scale and/or intense human exposure, even if it may be possible to require industry to perform the testing.

Most chemicals are nominated and selected for testing because toxicologic information is lacking and because the potential exists for human exposure. Other important criteria include production levels, physical and chemical properties, agency interests, and significance to society. The NTP toxicology testing strategy: identify with assurance the major toxic effects for each chemical studied. This includes (in addition to identifying chemical mutagens and carcinogens) damage to critical target organs such as the reproductive system, lungs, liver, and nervous system.

Nominations of chemicals for toxicological testing are submitted by the NTP participating agencies as well as other government agencies, industry, labor, and the public. The nominating source is asked to submit the name of the chemical, the particular toxicological tests desired, the rationale for testing, and to provide the available background data on production, use, exposure, environmental occurrence, and toxic properties in a supporting summary document.

An initial examination determines which proposed chemicals have already been tested, are being tested, are scheduled for test, or have been previously considered and rejected for testing by the NTP or its predecessors.

Literature containing relevant data is assessed and literature summaries are prepared for each chemical. Included in each literature summary are sections on chemical identification and physical properties, surveillance index (production, use, environmental occurrence, and available regulatory status and exposure limits), human exposure and health effects, research hypothesis to be tested, categories of study, and source of and reason(s) for nomination. Chemicals nominated for mutagenicity testing are reviewed only with regard to the available genetic toxicology information.

These summaries are reviewed and evaluated by the Chemical Evaluation Committee (composed of representatives from CPSC, EPA, FDA, OSHA, NCI, NCTR, NIEHS, NIOSH, and NTP)* who recommend the type(s) of testing to be considered. All suggestions and recommendations for future research and testing activities must satisfy at least one of the eight NTP principles of chemical selection.

Announcements listing the chemicals and the recommended types of testing appear in the *Federal Register* and the *NTP Technical Bulletin.* These notices solicit comments as well as information on completed, ongoing, and planned testing in the private sector. These steps are taken to encourage others outside the immediate program to participate in the NTP evaluation and selection process, as well as to specifically prevent unnecessary duplication. Revised summaries with additional public input are forwarded to the Board of Scientific Counselors for review. The Board evaluates these data and makes recommendations to the Executive Committee.

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*CPSC = Consumer Product Safety Commission; EPA = Environmental Protection Agency; OSHA = Occupational Safety & Health Administration; NCI = National Cancer Institute; NCTR = National Center for Toxicological Research; NIEHS = National Institute of Environmental Health Sciences; NIOSH = National Institute for Occupational Safety and Health; and NTP = National Toxicology Program.

Final summaries are submitted to the NTP Executive Committee who decide whether to select, defer, or reject the chemicals for testing. Following Executive Committee action, the chemicals are referred by the NTP Steering Committee to one or more participating agencies within the NTP: NIEHS, NIOSH, NCTR. At this stage certain approved chemicals may be identified as being inappropriate candidates for testing as a result of technical or budgetary reasons or in some cases public information describing ongoing testing may only have been submitted following Executive Committee decision. Such chemicals are then returned to the Executive Committee for reconsideration.

All chemicals selected are then tested as time and resources permit.

Toxicology and Carcinogenesis Bioassay

The "standard" two-year carcinogenesis bioassay remains as the most definitive method for detecting chemical carcinogens in animals. The standard protocol as developed by the NCI (and frequently still used by the NTP) typically uses two rodent species (usually Fischer 344 rats and B6C3F$_1$ mice), both sexes, and administration of multiple dose levels (concurrent controls, low dose, and high dose) of a chemical to groups of 50 animals, beginning at weaning and ending after two years. These experiments are designed primarily to determine whether selected chemicals produce cancer in animals. Chemicals tested in the NTP Carcinogenesis Bioassay Program are chosen primarily on the bases of human exposure, available (or lack of) toxicology data, level of production, and chemical structure. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

Negative results, in which the test animals do not have a greater incidence of cancer than control animals, do not necessarily mean that a test chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a test chemical is carcinogenic for animals under the conditions of the test and indicate that exposure to the chemical is a potential hazard to humans. The determination of the risk to humans from chemicals found to be carcinogenic in animals often requires a wider analysis which at present extends beyond the purview of these studies.

The results of the bioassay also serve as the reference base for the validation of short-term carcinogenesis assays. Two additional objectives have been identified as priority items: (1) to expand the bioassay experimental protocols to extend and better characterize the toxicologic profile of chemicals; and (2) to investigate, develop, and validate accurate, less costly, and more rapid methods for detecting carcinogenic potential.

Under the NTP, the carcinogenesis bioassay procedure(s) has been and continues to be changed to meet the objective of a broadened toxicologic characterization of chemicals and, further, to lead or stay abreast of advancing scientific developments. Prior to NTP involvement, the prechronic phases of the bioassay—which include single dose (acute), 14-day repeated dose, and 90- to 120-day repeated dose studies—were conducted to determine gross toxicity and general target organ effects at different dose levels as a primary basis for setting appropriate doses for the two-year bioassay studies. Now, the NTP has begun to gather routinely other information related to target organ effect: chemical disposition, fertility and reproduction, urinalysis, and hematology also are obtained from the prechronic studies—especially the 90-day study; certain other specific studies as applicable are included in the chronic two-year studies as well. Once those parameters that may be altered through exposure to the tested chemicals are identified, then suspect chemicals are referred to specific organ system groups for more detailed study of the functional, biochemical, and morphologic effects of the test compounds. Also, wider analysis of the quantitative and comparative absorption, distribution, metabolism, and excretion patterns may be desired. For instance, 28 (70%) of the 40 chemical starts in FY 1980 included specific toxicology studies in the prechronic testing phase. All chemicals started on test in FY 1981 had an expanded design including other select studies. Significantly all chemicals selected for chronic bioassay will be profiled for chemical disposition patterns. The goal is to ensure that all major toxic effects will be identified for each chemical being considered for long-term bioassays.

With this composite information base, the doses for the chronic study are selected. The high dose, termed the estimated maximum tolerated dose (EMTD), represents the highest dose of a chemical or substance given during a chronic study that can be predicted not to alter the treated animals' normal longevity from toxic effects other than carcinogenicity. The low dose ordinarily equals 1/2 EMTD. Other empirical factors include weight gain/food consumption data; for instance, a decrease in weight gain near 10-15% (not associated with a tumorigenic response) is often used as a general indication that the EMTD was achieved.

Prior to commencing the actual long-term carcinogenesis bioassay, all chemicals undergo genetic toxicology testing in at least five *in vitro* short-term assays: (1) gene mutations in backteria—*Salmonella typhimurium microsome;* (2) gene mutations in mammalian cells—mouse lymphoma (L5178Y, thymidine kinase); (3) chromosome damage in mammalian cells—cytogenetic damage and sister chromatid exchange (*in vitro*, CHO); (4) a mammalian cell transformation assay—(BALB/c -3T3); and (5) a direct measure of DNA damage/repair (which does not necessarily result in mutation or transformation)—unscheduled DNA synthesis (rat hepatocytes). These data, together with other prechronic bioassay information, are used by the experimental design groups for preparing appropriate study protocols and are used by staff for assisting in establishing priorities for chemicals queued into the long-term carcinogenesis bioassay. A key decision that must be made at this juncture between the completion of the prechronic phase and the beginning of the chronic study centers directly on whether indeed the lifetime bioassy should be done at all.

Thus, while the lifetime animal bioassay remains the best procedure for determining the carcinogenic potential of chemicals, NTP does not ordinarily use a standardized design. Rather the design is adapted to the special testing needs identified for the particular chemical. The NTP tailors its testing protocols to the particular chemicals based on the results from the prechronic testing phases, on available literature, and on structure-activity relations. These new protocols permit better, more specific information to be generated for the tested compounds, which increases the effectiveness of the tests for potential human risk estimations. Such protocols also will be useful as guidelines for testing undertaken by other agencies and by industry. As examples, the NTP continues to pursue actively other design methodologies—increase the number of dose levels, "unbalanced" distribution of animals among dose groups, interim kills, and reduced essential histopathology.

Bioassay Results

During FY 1981, 23 long-term carcinogenesis bioassays were completed and the conclusions and data were evaluated and approved by the NTP staff review committee and subsequently by the external NTP peer review panel. Under the conditions of these carcinogenesis bioassays, 12 (52%) were considered negative, 10 (44%) positive, and 1 (4%) equivocal. One, polybrominated biphenyl (PBB) mixture (Firemaster FF-1), was conducted using a broadened experimental protocol aimed at providing a better dose-response determination (through use of six doses) and at giving more data on toxicologic end points other than induction of cancer. Nontumor toxicities of this PBB included porphyrogenic effects, decreases in serum thyroid hormones, hematotoxicity, enzyme alterations, and hepatotoxicity.

Since the NTP became actively involved in the carcinogenesis bioassay technical report review process, 40 experimental studies have been completed (with draft or final reports being issued): 20 (50%) positive, 14 (35%) negative, 4 (10%) equivocal, 2 (5%) inadequate; see Table 1.

As of February 1982: (1) Twenty-five chemicals have been newly assigned to the testing laboratories (Table 2). (2) Seventy-nine chemicals (89 separate studies) were in the prechronic testing

Table 2. Chemicals assigned to testing laboratories (February 1982).

Azodicarbonamide (inhalation)
o-Benzyl-*p*-chlorophenol
C.I. Acid Red 114
C.I. Direct Blue 15
C.I. Direct Blue 218
3,3′-Dimethoxybenzidine
3,3′-Dimethylbenzidine
N,*N*-Dimethylformamide
Ethylbenzene
Glutaraldehyde
N-Isopropyl-*N*′-phenyl-*p*-phenylenediamine
Isoproterenol hydrochloride
Manganese sulfate
Methdilazine (gavage)
Methdilazine (feed)
Methylphenidate
p-Nitroaniline
o-Nitroanisole
Propantheline bromide
Quercetin
Riddelliine
Triamterene
1,2,3-Trichloropropane
Tricresyl phosphate
Trimellitic anhydride
Turmeric, oleoresin (curcumin)

phase (Table 3); most include one or more additional toxicology studies in the prechronic testing phase. (3) Ninety-six chemicals frequent the chronic two-year testing stage (Table 4). There are a number of new or ongoing long-term carcinogenesis studies concerned with chemicals or mixtures of chemicals which humans encounter in occupational settings (benzene, diesel fuel marine, gentian violet, napththalene, Navy fuels JP-5, as examples). (4) Twenty-nine chemicals (35 separate studies) are in the histopathology phase (Table 5). (5) For 10 chemicals, reports are being written or draft reports are in program review (Table 6). Including e new chemical starts, there were 185 bioassay either the prechronic or the chronic bioassa testing phases at the end of FY 1981 (Tables 3 an . (6) In FY 1982, the NTP expects to complete rough peer review) 29 long-term bioassays (Ta 6 and 7).

Abstracted from the peer-reviewed proved draft technical reports, Table 1 contains sorted information about the particular chemica arcino-genesis study (CAS Registry Number, pe review date, use, route and dose, contract labora ry, and

Table 3. Chemicals in the prechronic testing phase (February 1982).

Acetonitrile
Allyl glycidyl ether
1-Amino-2,4-dibromoanthraquinone
Amphetamine sulfate
Azodicarbonamide (gavage)
Benzaldehyde
2,2-Bis(bromomethyl)-1,3-propanediol (gavage)
2,2-Bis(bromomethyl)-1,3-propanediol (dermal)
γ-Gamma-butyrolactone
β-Cadinene
Caffeine
Carbon disulfide
d-Carvone
Catechol
Chloramine
Chloramphenicol
Chloroacetophenone
p-Chloroaniline
o-Chlorobenzalmalononitrile
Chlorpromazine hydrochloride
1,8-Cineol (eucalyptol)
Cinnamaldehyde
C.I. Pigment Red 23
C.I. Pigment Red 3
Coumarin
2,4-Diaminophenol hydrochoride
4,4′-Diamino-2,2′-stilbenedisulfonic acid
2,3-Dibromo-1-propanol (gavage)
2,3-Dibromo-1-propanol (dermal)
Diethyl phthalate
3,4-Dihydrocoumarin
Dimethoxane
Dimethyloldihydroxyethyleneurea (gavage)
Dimethyloldihydroxyethyleneurea (inhalation)
Doxylamine
Epinephrine hydrochloride
1,2-Epoxybutane
Ethyl bromide
Ethyl chloride
Ethylenediamine
Ethylene glycol
Ethylenethiourea
Formaldehyde
Furan
Furfural
Furfuryl alcohol
HC Yellow 4
Hexachlorocyclopentadiene
Hexachloroethane
Hydroquinone
4-Hydroxyacetanilide
5-Hydroxytryptophan
Isobutyl nitrite
Mercuric chloride
Methapyriline (acidified H_2O)
Methapyriline (neutral H_2O)
6-Methylcoumarin
N-Methylolacrylamide
Monochloroacetic acid
Nitrobenzene (gavage)
Nitrobenzene (dermal)
Ochratoxin A
Palladium (II) chloride
Pentachloroanisole
Pentachlorophenol, Dowicide EC-7
Pentachlorophenol, DP-2
Pentachlorophenol, purified
Pentachlorophenol, technical
Phenolphthalein
Phenytoin
Polybrominated biphenyl (FF-1)
Polysorbate 80
Probenecid
Promethazine
Pyrilamine
p-Quinone
Resorcinol
Sodium azide
Tetrahydrofuran
Tetranitromethane
Thenyldiamine
Titanocene dichloride
Toluene, commercial (gavage)
Toluene, commercial (inhalation)
Tripelenamine
Tris(2-chloroethyl) phosphate
1-Vinyl-3-cyclohexene dioxide (gavage)
1-Vinyl-3-cyclohexene dioxide (dermal)
2,6-Xylidine

Table 4. Chemicals in the chronic testing phase (February 1982).

2-Ami -4-nitrophenol
2-Ami -5-nitrophenol
Ampic lin trihydrate
Benze
Benzo ran
Benzy alcohol
Benzy chloride
Boric id
Brom ichloromethane
Brom orm
1,3-Bu adiene
2-But one peroxide
t-Buty alcohol
n-But chloride
Casto oil
Chlor dic acid
Chlori ated trisodium phosphate
Chloro libromomethane
3-Chlo o-2-methylpropene
Chloro wax 40
Chloro wax 500C
Chlorpheniramine maleate
C.I. Acid Orange 3
C.I. Basic Red 9 (*p*-rosaniline)
C.I. Disperse Blue 1
Coconut oil acid diethanolamine
Decabromodiphenyl oxide
p-Dichlorobenzene
2,4-Dichlorophenol
Dichlorvos
Diesel fuel marine
Diethanolamine
Diglycidylresorcinol ether (DGRE)
N,N-Dimethylaniline
Dimethyl hydrogenphosphite
Dimethyl methylphosphonate
Dimethyl morpholinophosphoramidate
Dimethylvinylchloride
Diphenhydramine hydrochloride
Ephedrine sulfate
1,2-Epoxyhexadecane
Erythromycin stearate
Ethyl ne chlorohydrin
Ethyl ne glycol monoethyl ether
Ethyl ne oxide
Furos mide
Genti n violet
Gluta ldehyde
Glyci l
HC Blue 2
HC Red 3
Hexylresorcinol
Hydrochlorothiazide
Iodinated glycerol
Isophorone
Lauric acid diethanolamine
D-Limonene
Malonaldehyde
2-Mercaptobenzothiazole
8-Methoxypsoralen
α1-Methylbenzyl alcohol
Methyl carbamate
Methyl Dopa
Methylene chloride
Methyl methacrylate
Nalidixic acid
Navy fuels JP-5 (petroleum derived)
Nitrofurantoin
Nitrofurazone
Oleic acid diethanolamine
Oxytetracycline hydrochloride
Penicillin V potassium
Pentachloronitrobenzene
Pentaerythritol tetranitrate
Phenylbutazone
Phenylephrine hydrochloride
N-Phenyl-2-naphthylamine
o-Phenylphenol
Pyridine
Rhodamine 6G
Rotenone
Roxarsone
Sodium fluoride
Styrene oxide
Succinic anhydride
Sulfamethazine
Tetrachloroethylene
Tetracycline hydrochloride
Tetrakis(hydromethyl)phosphonium chloride (THPC)
Tetrakis(hydroxymethyl)phosphonium sulfate (THPS)
Trichlorfon
Tris(2-ethylhexyl) phosphate
4-Vinylcyclohexene
Vinyl toluene
Witch hazel
Xylenes
Xylene sulfonic acid, sodium salt

the NTP scientist responsible for design, implementation, interpretation, and report preparation) and the abbreviated results. This table includes summary information on the six chemical carcinogenesis bioassays evaluated in December 1981, on the 3 reported in FY 1981, on the nine completed and peer reviewed in June 1980, and on two considered inadequate (TCDD-DMBA dermal and asbestos-DMH oral studies). The letter "P" following the *Technical Report* (*TR*) number in column two means that the *NTP Technical Report* has been published; detailed condensations of four carcinogenesis bioassays so designated follow this article. The others settle into various stages of publica-

Table 5. Chemicals in the histopathology phase (February 1982).

Asbestos, amosite
Asbestos, chrysotile (SR)
Asbestos, chrysotile (IR)
Asbestos, crocidolite
Chlorobenzene
C.I. Acid Yellow 73
Diallyl phthalate (rats)
o-Dichlorobenzene
1,2-Dichloropropane
1,3-Dichloropropane
Diglycidylresorcinol ether (DGRE)
Dimethylbenzanthracene (DMBA)/
  tetradecanoyl phorbol acetate (TPA)
Dodecyl alcohol, ethoxylated
Ethyl acrylate
Gilsonite
HC Blue 1
8-Hydroxyquinoline
Methylene chloride
Mirex
Monuron
p-Nitrophenol
Propylene
Propylene oxide
Sodium dodecyl sulfate
Sodium (2-ethylhexyl) alcohol sulfate
Tetrachloroethylene
Tremolite
1,1,1-Trichloroethane
Trichloroethylene

Table 6. Chemicals in the draft report—program review process (February 1982).

Allyl isovalerate
Ascorbic acid
Benzyl acetate
Diallyl phthalate (mice)
Geranyl acetate
Melamine
4,4′-Methylenedianiline dihydrochloride
Tetracholoroethylene
2,4-Toluene diisocyanate and
  2,5-toluene diisocyanate
Trichloroethylene

Table 7. Chemicals for which carcinogenesis bioassays will be completed (through peer review) in FY 1982 (February 1982).[a]

Agaritine[b]
Asbestos, amosite
Asbestos, chrysotile (short range)
Asbestos, chrysotile (intermediate range)
Asbestos, crocidolite
Bis(2-chloro-1-methylethyl) ether (BCMEE)
  and 2-chloro-1-methylethyl
  (2-chloropropyl) ether
Dimethylbenzanthracene (DMBA)/
  12-O-tetradecanoylphorbol-13-acetate (TPA)
D-Mannitol
N-Nitrosodiethanolamine[b]
Propyl gallate
Rotenone
Sodium (2-ethylhexyl) alcohol sulfate
1,1,1,2-Tetrachloroethane
Tetrachloroethylene
Tremolite
Trichloroethylene
2,6-Xylidine
Zearalenone
Ziram

[a] Plus those ten chemicals listed in Table 6.
[b] Journal publication (no technical report).

tion; these will be featured in future carcinogenesis bioassay results articles from the National Toxicology Program.

Document Availability—To receive copies of the *NTP Annual Plan for Fiscal Year 1982* (and for FY 1981), the *NTP Technical Bulletin*, and the *NTP Technical Reports* on a particular chemical carcinogenesis bioassay study, write: Mr. S. d'Arazien, NTP Public Information Office, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709 (919-541-3991; FTS 8-629-3991).

Technical Information—For more detailed information about completed, ongoing, or planned carcinogenesis bioassay studies, contact: Ms. Joan Chase, National Toxicology Program, 3A06 Landow Building, 7910 Woodmont Avenue, Bethesda, Maryland 20205 (301-496-1152; FTS 8-496-1152).

Chemical Nominations—Persons or organizations wishing to nominate other chemicals for testing should send the name of the chemical, testing requested, rationale, and other supporting information to: Dr. D. Canter, Chemical Nominations, National Toxicology Program, Building 31, Room 2B55, Bethesda, Maryland 20205.

I thank Dr. Larry Hart for use of the preprint version of the *NTP Annual Plan for Fiscal Year 1982*; Dr. Joseph [illegible]seman for helping prepare and verify Table 1; Mr. Jeffrey Se[illegible]ig and Mr. R. Michael Rowley for developing the BIOTEXT software file (Table 1); Ms. Florence Jordan and Ms. Pamela [illegible]on for initiating and maintaining the BIOTEXT file; each list[illegible] Chemical Manager; and Ms. Gerry Wandrisco for assistance in preparing this manuscript.

Environmental Health Perspectives
Vol. 45, pp. 199-200, 1982

Caprolactam: Condensation of the Carcinogenesis Bioassay Technical Report*

Caprolactam (aminocaproic lactam, 2-oxohexamethylenimine, CAS No. 105-60-2), the monomer used in the production of nylon-6, was selected and testing initiated by the Carcinogenesis Testing Program, National Cancer Institute (now part of the National Institute of Environmental Health Sciences/National Toxicology Program) because nearly one billion pounds per year are produced, because of the widespread use in food packaging materials and clothing, and because no carcinogenicity studies had been done (*1*).

Nylon 6 resins/fibers are used extensively in carpets, knit fabrics, hosiery, thread, hairbrushes, replacement parts for automobiles and machinery, flotation devices, and food packaging. Highly soluble in water, caprolactam reportedly leaches from clothing made from polyamide fibers when soaked in simulated perspiration (*2*). Ferguson and Wheeler (*3*) reported irritation of the skin, eyes, nose, and throat in workers occupationally exposed to caprolactam dust or vapor at concentrations ranging from 10 to 100 ppm.

Methods

Male and female inbred Fischer 344 rats and male and female hybrid B6C3F$_1$ mice, obtained from the Frederick Cancer Research Center, were used in this study. Control and treated groups contained 50 animals of each sex and species. For 103 consecutive weeks all groups received Ralston Purina Laboratory Chow. Treated groups were fed this diet containing 3750 or 7500 ppm (rats) and 7500 or 15,000 ppm (mice) caprolactam (~ 100% pure).

This carcinogenesis bioassay was conducted from January 1977 to February 1979 at Litton Bionetics under a subcontract to Tracor Jitco (prime contractor for the testing program).

All animals that died during the study or that were killed at the end of the exposure period were subjected to a gross necropsy and a complete histopathological examination. Statistical analyses comparing survival and numbers of animals with specific site tumors were done with trend tests and pairwise comparisons (*4-7*). The study design conformed to the NCI Guidelines for carcinogen bioassay (*8*).

Results

Throughout the bioassay, mean body weight gains for treated rats and mice decreased (and dose related) in comparison to controls. For rats, feed consumption by high dose males and females was 70-80% of that for controls; no difference was observed for mice. Survival was comparable among all groups.

The incidences of caprolactam-treated animals with specific site tumors did not differ significantly from those diagnosed in controls. Tables 1 (rats) and 2 (mice) list those primary tumors occurring in at least three animals of any one group.

Discussion

Pituitary carcinomas in male rats showed a statistically significant ($p < 0.05$) positive linear trend (0/46 control, 0/49 low dose, 3/43 high dose); female rat incidences were 2/49, 1/49, 1/47. Comparison of treated groups with controls revealed no statistical difference; likewise when adenomas were combined with carcinomas. This response was not considered associated with caprolactam administration. Other

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Single copies of the complete technical report (TR 214) may be obtained from the Public Information Office, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

Table 1. Primary tumors in male and female F344 rats fed diets containing caprolactam.

Tumor	Males			Females		
	Control	3750 ppm	7500 ppm	Control	3750 ppm	7500 ppm
Adrenal cortical adenoma	1/49	0/50	0/49	4/48	3/50	[illegible]/50
Adrenal pheochromocytoma	10/49	8/50	7/49	2/48	5/50	[illegible]/50
Hepatocellular carcinoma	1/50	3/49[a]	2/50	0/48	0/50	[illegible]/50
Leukemia	13/50	11/50	16/50	9/49	10/50	[illegible]/50
Mammary gland	3/50	4/50	1/50	12/49	7/50	[illegible]/50
Pituitary adenoma or carcinoma	10/46	11/49	11/47	24/49	24/49	[illegible]/47
Subcutaneous fibroma or fibrosarcoma	6/50	4/50	1/50	1/49	0/50	[illegible]/50
Testicular interstitial cell	41/49	43/50	48/50	–	–	–
Thyroid C-cell adenoma	3/46	1/45	5/49[b]	2/44	4/46	[illegible]/46
Thyroid follicular cell carcinoma	0/46	0/45	0/49	0/44	1/46	[illegible]/46[c]
Uterine endometrial stromal polyp	–	–	–	12/49	20/50	[illegible]/50

[a] Two other male rats had neoplastic nodules.

[b] One other male rat had a thyroid C-cell carcinoma.

[c] One other female rat had a thyroid follicular-cell adenoma.

Table 2. Primary tumors in male and female B6C3F$_1$ mice fed diets containing caprolactam.

Tumor	Males			Females		
	Control	3750 ppm	7500 ppm	Control	3750 ppm	7500 ppm
Circulatory system hemangiosarcoma	0/50	0/50	0/50	3/50	0/49	1/50
Hepatocellular adenoma	3/50	1/50	4/49	0/50	0/49	1/50
Hepatocellular carcinoma	5/50	9/50	6/49	1/50	1/49	0/50
Leukemia	0/50	0/50	1/50	4/50	2/49	0/50
Lymphoma	9/50	6/50	5/50	17/50	21/49	12/50
Lung alveolar/bronchiolar adenoma	3/50	3/50	2/49	3/50	0/49	0/50
Lung alveolar/bronchiolar carcinoma	1/50	2/50	2/49	0/50	0/49	0/50

effects interpreted as being unrelated to the administration of caprolactam included the negative trends calculated for papillary adenocarcinomas of the mammary gland in female rats (4/49, 1/50, 0/50), for subcutaneous fibromas in male rats (5/50, 3/50, 0/50), for lung alveolar/bronchiolar adenomas in female mice (3/50, 0/49, 0/50), and for leukemias/lymphomas of the hematopoietic system in female mice (4/50, 2/49, 0/50).

Because no evidence of neoplastic or nonneoplastic lesions could be related to dietary administration of caprolactam, under the conditions of this bioassay caprolactam was not carcinogenic for F344 rats or B6C3F$_1$ mice of either sex (*9*).

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Environmental Health Perspectives
Vol. 45, pp. 201-203, 1982

Cytembena: Condensation of the Carcinogenesis Bioassay Technical Report*

Cytembena (cytembene; 2-butenoic acid; 3-bromo-4-(4-methoxyphenyl)-4-oxo-, sodium; NSC 104801; CAS No. 21739-91-3) was selected for testing by the Carcinogenesis Testing Program, National Cancer Institute (now part of the National Institute of Environmental Health Sciences/National Toxicology Program) because preliminary clinical results from Czechoslovakia indicated that this chemical might have use as a cytostatic agent (*1-4*). Clinical trials in the United States were discontinued because no apparent antineoplastic effects were seen (*5-7*).

Methods

Male and female inbred Fischer 344 rats and male and female hybrid $B6C3F_1$ mice, obtained from Frederick Cancer Research Center, were used in this study. Control and treated groups comprised 50 animals of each species and sex. For 104 consecutive weeks all groups were fed Wayne Lab Blox. Three times per week treated groups were given intraperitoneal injections of 7 or 14 mg/kg (for rats) and 12 or 24 mg/kg (for mice) of cytembena (99.4% pure). Control groups received 0.9% saline injections.

This carcinogenesis bioassay was conducted during January 1977 to January 1979 by Southern Research Institute under a subcontract to Tracor Jitco (prime contractor for the testing program).

All animals that died during the study or that were killed at the end of the exposure period were subjected to a gross necropsy and a complete histopathological examination. Statistical analyses comparing survival and numbers of animals with specific site tumors were done with trend tests and pairwise comparisons (*8-11*). The study design conformed to the NCI Guidelines for carcinogen bioassay (*12*).

Results

Mean body weights of dosed and vehicle-control rats were comparable throughout the bioassay. Mean body weights of dosed and vehicle-control mice were comparable for the first 73 weeks of the bioassay; mean body weight of the high dose male mice was slightly lower than that of the vehicle controls after 73 weeks, and that of the high dose female mice was lower after week 87.

Survival for male rats was significantly decreased ($p < 0.001$) in the dosed groups relative to controls (29/50 control, 12/50 low dose, 12/50 high dose), whereas survival rates for female rats were comparable (30/50, 33/50, 28/50). All male mice groups exhibited markedly decreased survival compared to historic rates (20/50, 19/50, 12/50); female mice survival was comparable among groups (41/50, 33/50, 34/50).

Increased tumor incidences were diagnosed in male rats for mesotheliomas and in female rats for mammary gland fibroadenomas (Table 1).

The numbers of cytembena-treated rats with other specific site tumors and the frequencies of cytembena-treated mice with lesions did not differ significantly from those observed in controls. Tables 2 (rats) and 3 (mice) list those primary tumors found in at least three animals of any one group.

Discussion

The increased number of treated male rats with mesotheliomas in the tunica vaginalis and in multiple organs clearly associates these lesions with cytembena administration (Table 1). Malignant

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Single copies of the complete technical report (TR 207) may be obtained from the Public Information Office, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

Table 1. Primary tumor increases in male and female F344 rats given cytembena by intraperitoneal injecti

	Male			Female		
Tumor	Vehicle control	7 mg/kg	14 mg/kg	Vehicle control	7 mg/kg	1 /kg
Mammary gland fibroadenomas[a]	0/50	2/50	2/50	13/49	22/50	0[b]
Mesothelioma mesentery	0/50	3/50	0/50	0/49	0/50	0
Mesothelioma multiple organs[c]	3/50	26/50[b]	26/50[b]	0/49	0/50	0
Mesothelioma tunica vaginalis[d]	0/50	11/50[b]	10/50[e]	–	–	

[a] Significant dose-related trend for females ($p < 0.001$).
[b] Significantly greater than controls ($p < 0.001$).
[c] Significant dose-related trend for males ($p < 0.001$).
[d] Significant dose-related trend for males ($p < 0.05$).
[e] Significantly greater than controls ($p < 0.005$).

Table 2. Primary tumors in male and female F344 rats given cytembena by intraperitoneal injection.

	Male			Female		
Tumor	Vehicle control	7 mg/kg	14 mg/kg	Vehicle control	7 mg/kg	14 mg/kg
Adrenal pheochromocytoma	12/50	7/50	7/49	3/49	2/50	1/50
Hepatocellular neoplastic nodule	1/49	1/50	1/50[a]	0/49	1/50	4/50
Leukemia	20/50	12/50	22/50	8/49	8/50	8/50
Lymphoma	0/50	0/50	0/50	0/49	3/50	1/50
Mesentery lipoma	0/50	2/50	2/50	0/49	0/50	3/50[b]
Pancreatic islet-cell adenoma or carcinoma	3/50	5/50	4/49	0/49	1/48	1/49
Pituitary adenoma	8/50	3/50	5/50	14/47	13/46	16/47
Subcutaneous fibroma or fibroadenoma	3/50	0/50	2/50	0/49	0/50	2/50
Testicular interstitial cell	47/50	41/50	47/50	–	–	–
Thyroid C-cell adenoma or carcinoma	5/50	4/49	4/50	8/49	6/48	8/49
Thyroid follicular-cell adenoma or carcinoma	3/50	1/49	3/50	1/49	2/48	2/49
Uterine endometrial stromal polyp	–	–	–	16/49	11/50	11/50

[a] One other male rat had a hepatocellular carcinoma.
[b] One other female rat had a sarcoma, NOS (not otherwise specified).

Table 3. Primary tumors in male and female B6C3F$_1$ mice given cytembena by intraperitoneal injection.

	Male			Female		
Tumor	Vehicle control	12 mg/kg	24 mg/kg	Vehicle control	12 mg/kg	24 /kg
Circulatory system hemangiosarcoma	1/50	3/50	1/50	0/50	1/50	0
Harderian gland adenoma	2/50	3/50	1/50	1/50	0/50	0
Hepatocellular adenoma	6/49	5/49	6/50	0/50	0/48	9
Hepatocellular carcinoma	10/49	13/49	7/50	3/50	3/48	9
Lymphoma	5/50	4/50	3/50[a]	8/50	8/50[a]	0[b]
Lung alveolar/bronchiolar adenoma or carcinoma	6/50	7/47	5/49	7/50	4/49	8
Pituitary adenoma	0/47	0/42	0/44	1/47	3/46	44
Pituitary carcinoma	0/47	0/42	0/44	1/47	0/46	44
Thyroid follicular-cell adenoma	1/44	0/44	2/46	3/49[c]	2/48	19[c]
Uterine endometrial stromal polyp or sarcoma	–	–	–	0/49	3/48	2/50

[a] One other animal had leukemia.
[b] Two other females had leukemia.
[c] One other female had a thyroid follicular-cell carcinoma.

mes heliomas involved the serosal surfaces of most of the abdominal organs including the spleen, panc eas, mesentery, urinary bladder, seminal vesicles prostate, testicles (tunica vaginalis and epididy is), peritoneum, abdominal wall, and diaphra m. Grossly, abdominal distention was frequently obse ved. This distention resulted from accumulation f large quantities of dark brown peritoneal fluid Most serous surfaces manifested a rough, gran lar appearance. The mesentery was usually thicl ened and nodular.

H tologically, most mesotheliomas were of the papi ary type. There were numerous villous or papi ary projections on the serous surface of affected orga s. The stromal element of the villi consisted of conn ctive tissue cells. The villi or projections were cove ed by prominent mesothelial cells. The cell nucl i had moderately dense stippled chromatin and were uniformly round to oval. Most nuclei had one or two small nucleoli. The cytoplasm was distinct and abundant. Mitotic figures were commonly seen. There was frequent invasion of the abdominal wall and diaphragm. Invasive cells were usually cuboidal or polygonal and sometimes formed pseudoglandular acini. These cells were usually more pleomorphic and were arranged in solid nests or cords.

Cytembena induced an increased incidence of female rats with proliferative and neoplastic lesions of the mammary gland. A high incidence of atypical mammary gland fibroadenomas was observed in the female rats. These neoplasms were characterized by numerous cystic ducts with extensive periductular fibrosis and cystic glandular hyperplasia. Many of the ducts contained numerous intraductular papillary growths. Epithelial cells covering the papillary proj ctions often showed piling up of cell nuclei. In som areas, there were dilated periductular acini. The epithelial cells lining these spaces stained deep ly basophilic and frequently showed a loss of norm al cellular orientation or polarity. Some mitotic acti ity was observed in these areas. The variable degr es of cellular atypia were suggestive of early mali nant transformation or change. These lesions repr sented a variant of mammary fibroadenomas. The e was also an increase in the number of cystic duct , cystic hyperplasia, epithelial hyperplasia, and obular hyperplasia in the mammary tissue of dose l female F344 rats.

P sitive dose-related trends were observed for fem le rats with hepatocellular neoplastic nodules (p 0.05) and for female rats with mesentery lipo a ($p < 0.05$). Pairwise comparisons between treated and control groups did not yield any significant differences. These borderline effects were not considered compound induced. Similarly, a significant trend ($p < 0.05$) was calculated for female mice with hepatocellular adenoma; no differences were found between treated groups and controls or when liver tumors (adenomas and carcinomas) were considered together.

In conclusion, under the conditions of this bioassay, cytembena was carcinogenic for male and female F344 rats, causing increased numbers of males with mesotheliomas in the tunica vaginalis and in multiple organs and causing increased frequencies of females with fibroadenomas in the mammary glands. Cytembena was not carcinogenic for B6C3F$_1$ mice of either sex (*13*).

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Environmental Health Perspectives
Vol. 45, pp. 205-207, 1982

Di(2-ethylhexyl) Adipate: Condensation of the Carcinogenesis Bioassay Technical Report*

Di(2-ethylhexyl) adipate [bis(2-ethylhexyl) adipate, DEHA, octyl adipate, dioctyl adipate, DOA, CAS No. 03-23-1], a plasticizer added to vinyl plastics to give low temperature flexibility, is dispersed (not bound) in the polymer chain matrix. Approved by the U.S. Food and Drug Administration for use in plastics that may contact nonfatty, nonalcoholic foods (not to exceed 24% by weight of the plastic polymer), DEHA has wide use in vinyl packaging film for refrigerated and frozen food products. Other products containing DEHA include electric wire insulation, garden hoses, vinyl coated fabrics for automotive and upholstery use, synthetic rubber, base oils for hydraulic fluids, and, among others, polyvinyl tubing for hemodialysis. For these uses 44 million pounds were produced in 1978.

DEHA was selected as a representative of the adipate class of plasticizers, because it is structurally related to di(2-ethylhexyl) phthalate, because human exposure is widespread, and because no carcinogenesis studies had been done. Testing was initiated by the Carcinogenesis Testing Program, National Cancer Institute (now part of the National Institute of Environmental Health Sciences/National Toxicology Program).

Methods

Male and female inbred Fischer 344 rats and male and female hybrid B6C3F$_1$ mice, obtained from the Frederick Cancer Research Center, were used in this bioassay. For 103 consecutive weeks all groups received powdered Wayne Lab Blox. Treated groups were fed this diet containing 12,000 ppm or 25,000 ppm di(2-ethylhexyl) adipate (> 98% pure).

This carcinogenesis bioassay was conducted between April 1977 and May 1979 at EG&G Mason Research Institute under a subcontract to Tracor Jitco (prime contractor for the testing program).

All animals that died during the study or that were killed at the end of the exposure period were subjected to a gross necropsy and a complete histopathological examination. Statistical analyses comparing survival and numbers of animals with specific site tumors were done with trend tests and pairwise comparisons (*1-4*). The study design conformed to the NCI Guidelines for carcinogen bioassay (*5*).

Results

Mean body weights of high dose male and female rats and treated male and female mice were lower than the corresponding controls. Survival was comparable among all groups of mice and male rats; survival in the female control rats was reduced relative to the treated groups (29/50 control, 39/50 low dose, 44/50 high dose).

The number of DEHA-treated rats with specific site tumors did not differ significantly from those found in controls. Table 1 lists those primary tumors occurring in at least three animals of any one group.

Liver tumors occurred with significantly higher frequencies in treated male and female mice than in controls (Table 2). Other specific site tumors were not significantly increased in treated mice when compared to controls (Table 3).

Discussion

In male rats the positive dose-related trend (p = 0.010) and the statistically significant ($p < 0.05$) increase in interstitial cell testicular tumors in the

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Single copies of the complete technical report (TR 212) may be obtained from the Public Information Office, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

Table 1. Primary tumors in male and female F344 rats fed diets containing di(2-ethylhexyl) adipate.

	Male			Female		
Tumor	Control	12,000 ppm	25,000 ppm	Control	12,000 ppm	25[illegible] ppm
Adrenal cortical adenoma	1/48	1/50	0/50	3/50	0/50	[illegible]8
Adrenal pheochromocytoma	6/48	3/50	1/50	1/50	2/50	[illegible]8
Hepatocellular carcinoma	0/49	1/50	2/50	0/49	1/50	[illegible]0
Hepatocellular neoplastic nodule	2/49	1/50	0/50	0/49	2/50	[illegible]0
Leukemia	9/49	11/50	8/50	12/50	5/50	[illegible]0
Mammary gland	1/49	2/50	0/50	14/50	6/50	[illegible]0
Mesothelioma	1/49	1/50	3/50	0/50	0/50	[illegible]0
Pituitary adenoma or carcinoma	4/44	3/47	3/44	21/47	25/48	[illegible]9
Preputial gland carcinoma	1/49	4/50	1/50[a]	–	–	
Skin squamous cell papilloma	1/49	4/50	1/50	0/50	0/50	[illegible]0
Subcutaneous fibroma	4/49	1/50[b]	2/50	0/50[b]	1/50	[illegible]50[c]
Testicular interstitial cell	43/49	47/50	49/49	–	–	
Thyroid C-cell carcinoma or adenoma	3/49	4/49	1/46	4/50	0/50	[illegible]47
Uterine endometrial stromal polyp	–	–	–	11/50	10/50	1[illegible]/50

[b] One other animal had a subcutaneous febrosarcoma.
[c] One female had a subcutaneous fibroadenoma.

Table 2. Primary liver tumor increases in male and female B6C3F$_1$ mice fed diets containing di(2-ethylhexyl) adipate.

	Males			Females		
Hepatocellular tumor	Control	12,000 ppm	25,000 ppm	Control	12,000 ppm	25,000 ppm
Carcinoma[a]	7/50	12/49	12/49[b]	1/50	14/50[c]	12/49[d]
Adenoma[e]	6/50	8/49	15/49[f]	2/50	5/50	6/49
Carcinoma or adenoma[g]	13/50	20/49	27/49[d]	3/50	19/50[c]	18/49[c]

[a] Significant dose-related trend for females ($p < 0.005$).
[b] One other male had a neoplasm, NOS (not otherwise specified).
[c] Significantly greater than controls ($p < 0.001$).
[d] Significantly greater than controls ($p < 0.005$).
[e] Significant dose-related trend for males ($p < 0.05$).
[f] Significantly greater than controls ($p < 0.05$).
[g] Significant dose-related trend for males and females ($p < 0.005$).

Table 3. Primary tumors in male and female B6C3F$_1$ mice fed diets containing di(2-ethylhexyl) adipate.

	Males			Females		
Tumor	Control	12,000 ppm	25,000 ppm	Control	12,000 ppm	25[illegible]pm
Circulatory system: angiosarcoma, hemangioma, hemangiosarcoma	2/50	2/50	1/50	3/50	3/50	[illegible]
Lymphoma	16/50	7/50	6/49	23/50	14/50	[illegible]
Lung alveolar/bronchiolar adenoma	8/50	9/49	3/49	5/49[a]	1/49	[illegible]
Pituitary adenoma	0/40	0/31	0/36	8/39	6/37	[illegible]
Subcutaneous fibroma or fibrosarcoma or sarcoma, NOS	5/50	3/50	0/49	2/50[b]	0/50	[illegible]

[a] One other female rat had an alveolar/bronchiolar carcinoma.
[b] One other female rat had a neoplasm, NOS (not otherwise specified).

high dose group were not considered compound related since most (> 80%) aging F344 male rats have this lesion.

Significant negative trends were obtained for male rats with adrenal gland pheochromocytomas ($p < 0.05$) and for female rats with adrenal cortical adenomas ($p < 0.05$) and with mammary gland fibroadenomas ($p = 0.001$).

Hepatocellular carcinomas were increased [illegible] both low and high dose female mice; hepatocellular adenomas or carcinomas combined occurred [illegible] high dose mice of either sex and in low dose female mice at incidences that were dose related and significantly higher than those in the controls. The time to observation of hepatocellular adenomas or carcinomas in the dosed female mice, but not in dosed male mice was significantly shorter than the time to observation of these tumors in the controls. Because the increase in liver tumors in males reflects only an increase in adenomas for the high dose group and because the time to observation of tumors in dosed groups as compared with the control group was not significantly different, the association of liver tumors in the males with administration of di(2-ethylhexyl) adipate is not considered conclusive.

Hepatocellular adenoma compressed the adjacent liver tissue. Cells in the adenoma were large. Cytoplasm of the cells was acidophilic or vacuolated and nuclei were hyperchromatic. Hepatocellular carcinoma involved a part or an entire lobe of the liver. The lobular architecture was distorted and cell plates were two or more cells thick, forming trabeculae. Cellular pleomorphism was apparent. The nuclei had coarse chromatin, and the nucleoli were prominent. Both normal and abnormal mitotic figures were numerous. Areas of necrosis and mineralization were common in the large tumors.

Hepatocellular carcinoma metastasized to the lung in 14 male mice (control, 5; low dose, 4; high dose, 5) and in 11 female mice (low dose, 6; high dose, 5). In all cases, the primary liver tumors were of the trabecular type. The other sites of metastases were the kidney, adrenal, and lymph nodes in dosed female mice.

The historical incidence of liver tumors in male $B6C3F_1$ mice at this laboratory is: adenomas, 35/398 (9%, range 0-16%); carcinomas, 86/398 (22%, range 14-30%); combined tumors, 116/398 (29%, range 18-36%). The historical incidence of tumors in female $B6C3F_1$ mice at this laboratory is: adenomas, 18/397 (5%, range 0-18%); carcinomas, 14/397 (4%, range 0-8%); combined tumors, 31/397 (8%, range 2-20%).

Negative trends were calculated for male (p = 0.010) and for female (p = 0.001) mice with lymphomas; the number of mice in each treated group with lymphoma was significantly less ($p < 0.05$) than the corresponding controls. This interesting phenomenon of decreases in leukemias/lymphomas concomitant with increases in hepatocellular neoplasms is receiving considerable research attention from the National Toxicology Program (*6*).

Di(2-ethylhexyl) adipate was not mutagenic for *Salmonella typhimurium* TA 1535, TA 1537, TA 1538, TA 98, and TA 100, with and without activation, or for *Saccharomyces cerevisiae* (*7*). Di(2-ethylhexyl) adipate is teratogenic for Sprague-Dawley rats (*8*) and causes dominant lethal mutations in ICR mice (*9*).

In conclusion and under the conditions of this bioassay, di(2-ethylhexyl) adipate was not carcinogenic for F344 rats of either sex. Di(2-ethylhexyl) adipate was carcinogenic for female $B6C3F_1$ mice causing increased numbers of female mice with hepatocellular carcinomas, and was probably carcinogenic for male $B6C3F_1$ mice, causing increased numbers of male mice with hepatocellular adenomas as well as inducing increased numbers of male mice with combined hepatocellular adenomas or carcinomas (*10*).

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Environmental Health Perspectives
Vol. 45, pp. 209-210, 1982

FD&C Yellow No. 6: Condensation of the Carcinogenesis Bioassay Technical Report*

FD&C Yellow No. 6 (Sunset Yellow FCF, CAS No. 2783-94-0), a water-soluble monoazo dye, has been used since 1929 to impart a reddish-yellow color to various gelatin desserts, sherbets, carbonated beverages, candies, cereals, jams, pickles, smoked fish, puddings, medicinals (liquids, tablets, and capsules), toothpastes, hair rinses, and other foods, drugs, and cosmetics.

The Carcinogenesis Testing Program, National Cancer Institute (now part of the National Institute of Environmental Health Sciences/National Toxicology Program) selected and initiated a carcinogenesis bioassay on FD&C Yellow No. 6 because this chemical has wide use as a food colorant and in myriad other consumer products, indicating low level yet repeated exposure to a sizable population. Further, results available from other studies were considered inadequate for conclusive evaluation due to the small numbers of animals, low dose levels, and short exposure periods (*1-4*).

Methods

Male and female inbred Fischer 344 rats and male and female hybrid B6C3F$_1$ mice, obtained from the Frederick Cancer Research Center, were used in this study. Control and treatment groups contained 50 animals of each sex and species, except for the control groups for rats, which consisted of 90 males and 90 females. For 103 consecutive weeks all groups received Ralston Purina Laboratory Chow meals. Treated groups were fed this diet containing 1.25% (12,500 ppm) or 2.5% (25,000 ppm) FD&C Yellow No. 6 (92% pure).

This carcinogenesis bioassay was conducted during December 1976–January 1979 at the Battelle Columbus Laboratories under a subcontract to Tracor Jitco (prime contractor for the program).

All animals that died during the study or that were killed at the end of the exposure period were subjected to a gross necropsy and a complete histopathological examination. Statistical analyses comparing survival and numbers of animals with specific site tumors were done with trend tests and pairwise comparisons (*5-8*). The study design conformed to the NCI Guidelines for carcinogen bioassay (*9*).

Results

Mean body weights of high dose female rats and all low dose groups were comparable to the controls. High dose male rats and high dose male and female mice weighed somewhat less (10% or less) than the controls. Survival was comparable among all groups.

The incidences of FD&C Yellow No. 6-treated animals with specific site tumors did not differ significantly from those observed in controls. Tables 1 (rats) and 2 (mice) list those primary tumors occurring in at least three animals of any one group.

Discussion

Hepatocellular carcinomas occurred in low dose (12,500 ppm) male mice at an incidence significantly higher than that in the controls (13/50, 26%; 22/48, 46%; $p < 0.05$; 16/50, 32%); departure from linear trend was likewise evident ($p < 0.05$), due mainly to a higher incidence in the low dose group. The historical control incidence (868/3,543, 24%) is sim-

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Single copies of the complete technical report (TR 208) may be obtained from the Public Information Office, National Toxicology Program, P. O. Box 12233, Research Triangle Park, North Carolina 27709.

Table 1. Primary tumors in male and female F344 rats fed diets containing FD&C Yellow No. 6.

Tumor	Males			Females		
	Control	12,500 ppm	25,000 ppm	Control	12,500 ppm	25,0 pm
Adrenal cortical adenoma	3/89	2/50	0/49	6/86	2/50	
Adrenal pheochromocytoma	14/89	3/50	11/49	4/86	1/50	
Hepatocellular neoplastic nodule	5/90	6/50	1/50	3/88	3/50	
Leukemia	22/90	12/50	17/50	16/88	12/50	1
Mammary gland	2/90	1/50	4/50	22/88	9/50	1
Pancreatic islet-cell adenoma or carcinoma	3/88	4/49	2/49	1/83	1/50	
Pituitary chromophobe adenoma or carcinoma	5/84	5/46	4/46	30/83	18/45	1
Sucutaneous fibroma or fibrosarcoma	5/90	2/50	3/50	2/88	0/50	
Testicular interstitial cell	86/90	48/50	50/50	–	–	
Thyroid C-cell carcinoma	2/89	4/49	4/50	3/86	2/50	2
Uterine endometrial stromal polyp	–	–	–	9/87	6/49	7

Table 2. Primary tumors in male and female $B6C3F_1$ mice fed diets containing FD&C Yellow No. 6.

Tumor	Males			Females		
	Control	12,500 ppm	25,000 ppm	Control	12,500 ppm	25,000 ppm
Lymphoma	9/50	1/49	10/50	15/50	5/50	10/49
Lung alveolar/bronchiolar adenoma or carcinoma	6/50	4/48	3/50	0/50	1/50	1/48
Hepatocellular adenoma	1/50	1/48	0/50	1/48	3/50	0/48
Hepatocellular carcinoma	13/50	22/48	16/50	7/48	0/50	4/48
Pituitary chromophobe adenoma or carcinoma	0/43	0/40	0/37	0/46	4/43	1/35
Skin and subcutaneous fibroma or sarcoma	5/50	5/49	6/50	0/50	0/50	0/49

ilar to the control rate in this study. Because the incidence of hepatocellular carcinomas in the 25,000 ppm group was not statistically different from controls and because the liver tumor occurrence in male $B6C3F_1$ mice is variable, the increased number of males with liver carcinoma cannot be clearly related to administration of FD&C Yellow No. 6.

Similarly, no other neoplastic or nonneoplastic lesions were considered induced by or associated with the administration of FD&C Yellow No. 6. Therefore, under the conditions of this bioassay, there was no clear evidence of carcinogenicity of FD&C Yellow No. 6 in F344 rats or $B6C3F_1$ mice of either sex (*10*).

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Announcements

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Environmental Health Perspectives
Vol. 45, p. 213, 1982

ERRATA

Mortality and Cancer Rates among Workers in the Swedish PVC Industry. Environmental Health Perspectives, 41: 145-151 (1981)

Authorship of the article, "Mortality and Cancer Rates among Workers in the Swedish PVC Industry, which appeared in Volume 41, pp. 145-151 (1981) of Environmental Health Perspectives, was incorrectly listed as G. Molina, B. Holmberg, S. Elofsson, L. Holmlund, R. Moosing and P. Westerholm. Authorship should have been listed as B. Holmberg, S. Elofsson, L Holmlund, R. Moosing, G. Molina and P. Westerholm.

Statistical Limitations in Relation to Sample Size. Environmental Health Perspectives, 42: 15-21 (1981)

Some power calculations for Tables 4 and 5 in the paper, "Statistical Limitations in Relation to Sample Size," which appeared in Volume 42, pp. 15-21 (1981) of Environmental Health Perspectives, were done incorrectly. The errors affect none of the arguments or findings in the paper; however, corrected tables are reproduced below.

Table 4. Power calculation for linear model analyses of Example 2, assuming dose values, person-years at risk, and dose-response functions shown in Table 2. Values correspond to multiples of the person-year array in Table 2, and to the given dose array and the array divided by 10.

	Dose			Dose/10		
	Lin-quad	Linear	Pure quad	Lin-quad	Linear	Pure quad
Coeff. × 1,000,000	2.55	2.5	2.5	1.19	2.5	0.33
PY multiplier (power of 10)						
−1	0.377	0.384	0.363	0.070	0.091	0.056
−0.5	0.765	0.775	0.745	0.089	0.137	0.060
0	0.995	0.996	0.993	0.132	0.252	0.070
0.5	1.000	1.000	1.000	0.240	0.537	0.089
1	1.000	1.000	1.000	0.511	0.926	0.131

Table 5. Power calculations for pure-quadratic model analyses of Example 2, assuming dose values, person-years at risk, and dose-response functions shown in Table 2. Values correspond to multiples of the person-year array in Table 2, and to the given dose array and the array divided by 10.

	Dose			Dose/10		
	Lin-quad	Linear	Pure quad	Lin-quad	Linear	Pure quad
Coeff. × 1,000,000	0.0159	0.0149	0.016	0.0556	0.123	0.016
PY multiplier (power of 10)						
−1	0.376	0.367	0.372	0.069	0.089	0.056
−0.5	0.763	0.751	0.367	0.087	0.132	0.061
0	0.995	0.994	0.994	0.129	0.241	0.071
0.5	1.000	1.000	1.000	0.232	0.513	0.092
1	1.000	1.000	1.000	0.491	0.909	0.139

Errata for "Statistical limitations in relation to sample size," by Charles E. Land, Environmental Health Perspectives Vol. 42, pp. 15-21, December 1981.

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Book
Harper, R., Smith, E. C. B., and Land, D. B. Odour Description and Classification. American Elsevier, New York, 1968.

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NIH PUBLICATION
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DRINKING WATER DISINFECTANTS

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ENVIRONMENTAL HEALTH PERSPECTIVES

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service–National Institutes of Health
National Institute of Environmental Health Sciences

VOLUME 46, DECEMBER 1982

SUBSCRIPTION INFORMATION

Subscription rates are as follows: Single copies $8.00, foreign $10.00, domestic $45.00 per year, foreign $56.25 per year. The yearly subscription is for 6 regular issues. Please send check or money order, in advance to the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402.

The Secretary of Health and Human Services has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through August 30, 1984.

ENVIRONMENTAL HEALTH PERSPECTIVES is a publication of the National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services. Opinions expressed herein, however, are not necessarily endorsed nor shared by any of the above agencies.

DHHS publication No. (NIH) 82–218

Address communications to:

Editor, ENVIRONMENTAL HEALTH PERSPECTIVES
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Reprints of Articles Should Be Requested from the Principal Authors Unless Stated Otherwise

Print Schedule: Volume 47 will present the proceedings of the Symposium on Biological Tests in the Evaluation of Mutagenicity and Carcinogenicity of Air Pollutants with Special Reference to Motor Exhausts and Coal Combustion Products.

Volume 46, December 1982

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International Symposium on Health Effects Of Drinking Water Disinfectants and Disinfectant By-Products

Sponsored by the
U. S. Environmental Protection Agency

Cincinnati, Ohio
April 21-24, 1981

Environmental Health Perspectives
Vol. 46, pp. 1-6, 1982

Introduction: Evaluating the Benefits and Potential Risks of Disinfectants in Drinking Water Treatment

by Joseph A. Cotruvo*

Probably the most significant public health measure introduced within the last 100 years has been the introduction of chlorine widely as a disinfectant for drinking water. Of course, not only chlorine but other oxidants also have been used, like ozone and chlorine dioxide in other parts of the world to a much greater extent than in the United States. The benefits are great and obvious. The issue for this conference is the possibility that there may be some subtle disbenefits that relate to the widespread use of these oxidants. We must consider that these oxidant chemicals and their by-products and their degradation products are the most ubiquitous organic chemical contaminants in drinking water and the most significant in terms of quantity and population exposure.

In the United States more than 150 million people consume disinfected water. Milligram amounts of chlorine and milligram amounts of by-products are being added to the water in the course of water treatment. Because of our familiarity with chlorine and chloroform, there is a great tendency to not take it very seriously if there are some risks associated. I think we would be extremely upset if various organic chemicals were being detected in drinking water coming from industrial discharges at the concentration levels that we find trihalomethanes (THMs) and other organic chemicals which are derived from the disinfection process. There is no particular reason why one should treat them differently toxicologically, but there are reasons why one should treat them differently in the sense of ultimate benefit of the process that produced them relative to potential risks.

The benefits of oxidation and disinfection of drinking water are many and we do not have to defend that. But, what is the issue in our regulatory context is the possibility that there may be some disbenefits, and it is essential to quantify those disbenefits and to minimize them while maximizing the benefit side of the equation. The amazing thing is that in the approximately 100 years since disinfectants were introduced into water treatment, there is still so little understanding of the toxicology of these chemicals and their by-products. Remember, these are the most widely used chemicals in water treatment, and there is such a small amount of information available on the risks from long-term deliberate systematic exposures to low levels of these materials by hundreds of millions of people. It is safe to say, however, that the people in this room and their work in the last five years probably far exceeds all of the work that has been done in the past 100 years in trying to obtain a better understanding of those possible toxicological endpoints.

Our interest is in three kinds of substances. We are interested in the disinfectants themselves, be they chlorine, ozone, chloramines, iodine or chlorine dioxide, or any other of the particular oxidants. We are also interested in the degradation products of those substances which are retained in the water such as the chlorites, chlorates, hypochlorites, or iodates and whatever additional products there happen to be. We are also interested in the chemical reaction products of those very potent, chemically active substances with the other materials which are in the water. Work has tended to concentrate on the volatile and hydrophobic organic chemicals because of the analytical breakthroughs that have occurred in the last five to seven years, so now there is much more information on the low molecular weight chlorinated by-products than on any others. However, there is very little information on the oxygenated by-products and higher molecular weight or more hydrophylic chemicals, and these make up the greatest portion, by far. The

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* Criteria and Standards Division, Office of Drinking Water, Environmental Protection Agency, Washington, D.C.

presence of these oxidants in the water also alters the oxidation states of many of the other substances that are in the water, be they nitrate instead of nitrite, or higher oxidation states of metals, and many examples where the presence of an oxidant causes a particular chemical specie to be in the water other than would have been there. I suspect that there are many benefits to the disinfection process that we are not aware of apart from disinfection; not the least, for example the question of conversion of nitrites to nitrates in water. The nitrates are probably less toxic than the nitrites, and it just might be that the reason methemoglobinemia is not often detected in high nitrate communities that consume public water is the fact that the water is disinfected. It may be that the presence of oxidizing conditions, including control of reducing organisms, may be a factor in determining susceptibilities, particularly of infants and the risks from water as on exposure route.

One other item that should be examined much more extensively is the *in vivo* chemistry of these substances, particularly the oxidants themselves, and their degradation products after they have been ingested. Beyond investigations of the water chemistry and toxicology of these substances and their byproducts in water, what are the consequences when for example, free chlorine or hypochlorite or chlorite is ingested and combines with proteins, enzymes, hemoglobin, or other macromolecules? There are some important experiments to be performed, and interesting studies of mechanism and effects to be explored. I think that these are some of the things that are going to be examined as this work continues to progress.

Regulatory Activity

From the regulatory standpoint, which is what this discussion was supposed to present, our activities really began in 1974 when the Safe Drinking Water Act passed. One of the reasons the Safe Drinking Water Act passed was because of the great interest and concern about the identification of chlorinated by-products as a result of the use of chlorine in drinking water. The Safe Drinking Water Act provided a number of authorities that never existed before in the United States to control the quality of drinking water. Up until that time, the primary responsibility and the primary control over drinking water quality rested in the hands of the states, and, in fact, to a great degree in the hands of the local communities and the local water treatment plant operators. The idea of the law was to provide a more uniform level of drinking water quality protection throughout the United States regardless of location and regardless of size of community or financial circumstances.

The law gave EPA two authorities. One of them is to establish acceptable limits for contaminants in drinking water; these are maximum contaminants levels (MCLs). The other had to do with the possibility of requiring specific treatments to be used in drinking water in those cases where maximum contaminant levels were not appropriate. At that time and even now, there are no specific MCLs or treatment requirements that deal with disinfectants, per se. There is no disinfection requirement in the United States. The only requirement relative to drinking water and sanitation is the necessity of meeting biological quality standards, the coliform standards. As a matter of option, as a matter of concensus and often of necessity, or state rules, most drinking waters in the United States are chlorinated. There is no national rule that relates to chlorine. Not only is there not an MCL for residual chlorine, there is also not a treatment requirement that demands the use of chlorine. However, two years ago EPA issued a maximum contaminant level for trihalomethanes. The trihalomethanes were the most ubiquitous, most readily identifiable by-products of the chlorination process. The overall guiding principle of the law is that EPA should identify substances in water that may have an adverse effect on health and in general establish MCLs controlling those substances to the extent feasible, taking costs and other factors into consideration (Tables 1 and 2).

Trihalomethanes

I think there has been an inordinant fixation on the fact that the MCL has been established for trihalomethanes. In fact the premise for the regulation and the impetus for its development was to deal with the entire area of disinfection and the use of chlorine. It is only because trihalomethanes happen to be the most readily identifiable markers for the by-products of the chlorination process that the MCL for THM happened to be established. It could have been and it could be in the future modified to reflect other by-products that are produced or some other kind of measurement such as "organically bound halogen." THMs could well be an indicator of the total condition of the water as it is affected by the presence of chlorine.

Figure 1 is a very good illustration of this point. The tip of that iceberg is a very tiny portion of the total amount of by-products that are known to exist. Very few of them have been identified at this point. Trihalomethanes were the most obvious ones among the total organic halogen group. The trihalo-

Table 1. National interim primary drinking water regulations.

Entity	Level
Arsenic	0.05 mg/l.
Barium	1.0 mg/l.
Cadmium	0.010 mg/l.
Chromium	0.05 mg/l.
Lead	0.05 mg/l.
Mercury	0.002 mg/l.
Nitrate (as N)	10 mg/l.
Selenium	0.01 mg/l.
Silver	0.05 mg/l.
Fluoride	1.4 - 2.4 mg/l. (ambient temp)
Endrin	0.0002 mg/l.
Lindane	0.004 mg/l.
Methoxychlor	0.1 mg/l.
Toxaphene	0.005 mg/l.
2,4-D	0.1 mg/l.
2,4,5-TP Silvex	0.01 mg/l.
Coliform bacteria	< 1/100 ml.
Radium-226 + radium-228	5 pCi/l.
Gross alpha particle activity	15 pCi/l.
Beta particle and photon radioactivity	4 mrem (annual dose equivalent)
Turbidity	1 Tu (up to 5 Tu)
Trihalomethanes (the sum of the concentrations of bromodichloromethane, dibromochloromethane, tribromomethane (bromoform) and trichloromethane (chloroform)	0.10 mg/l.
Sodium monitoring and reporting	
Corrosion monitoring and distribution system composition	

Table 2. National secondary drinking water regulations.

Entity	Level
Chloride	250 mg/l.
Color	15 color units
Copper	1 mg/l.
Corrosivity	Noncorrosive
Foaming agents	0.5 mg/l.
Iron	0.3 mg/l.
Manganese	0.05 mg/l.
Odor	3 threshold odor number
pH	6.5 - 8.5
Sulfate	250 mg/l.
TDS	500 mg/l.
Zinc	5 mg/l.

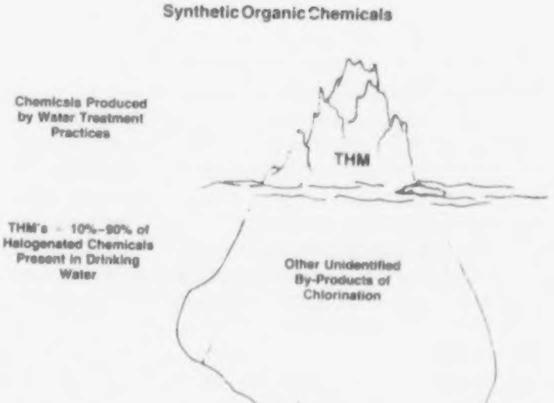
Synthetic Organic Chemicals
Chemicals Produced by Water Treatment Practices
THM
THM's ~ 10%–90% of Halogenated Chemicals Present in Drinking Water
Other Unidentified By-Products of Chlorination

FIGURE 1. Synthetic organic chemicals

methanes range from as little as 10% or so in some cases up to about 90% of the total organic halogens depending on the specific circumstances. So, obviously there is a great amount unknown on even just the chlorinated by-products or the brominated by-products. In addition, on a mass balance basis the organochlorine products produced during chlorination of water may only account for a few percent of the added chlorine itself and, in fact, the majority of the chemistry of chlorine in water probably is oxidation chemistry rather than chlorination chemistry. So there is a whole host of by-products for which there is very little information on their identity or toxicology, and which may contribute some increment of human risk. What must be determined, of course, is how significant is that risk and whether there are ways of minimizing it.

The THM regulation is very simple: 0.10 mg/l. for

Table 3. Summary of THM regulations.

Maximum contaminant level (MCL)	0.10 mg/l (100 μg/L) total trihalomethanes
Applicability	Community water systems that add disinfectant to the treatment process (ground and surface)
Effective:	
Systems > 75,000	2 years after promulgation
Systems 10-75,000	4 years after promulgation
Systems < 75,000	State discretion
Monitoring requirements:	Running annual average of a minimum of 4 samples per quarter per plant taken on same day
	Systems using multiple wells drawing raw water from a single aquifer may, with state approval, be considered one treatment plant for determining the required number of samples.
Effective:	
Systems > 75,000	1 year after promulgation
Systems 10-75,000	3 years after promulgation
Systems < 75,000	State discretion
Sample locations:	25% at extreme of distribution system; 75% at locations representatives of distribution.

the total THMs, and the implementation of that regulation is now in progress. The monitoring requirements in large communities (> 75,000) began (Table 3) in November 1980. The compliance requirements begin November 1981 and following that the sequence continues into smaller communities, down to 10,000 population. Smaller communities are dealt with by state determination.

But why regulate trihalomethanes? After reviewing all of the information, EPA felt there was no question that because of the widespread and extensive use of chlorine, there were a number of reasons why the by-products of chlorination should be controlled, at least by a reasonable first step in that process to reduce obviously unnecessary exposure. It is clear that water chlorination is the major route of exposure to the American population to chloroform and the other trihalomethanes. It is also clear that the most frequently found chemicals in water in almost every community are those trihalomethanes and the other by-products. The concentrations that are found in those waters are among the highest of any synthetic type chemicals that are detected. The risks that are associated with them are not established; they are, however, certainly suggested by the animal toxicology and to a degree by some of the human epidemiology that has been done. It is clear that we actually have a choice—that these chemicals are introduced deliberately and therefore, since they are introduced deliberately, means of control are within reach. The control technology is very common technology, so there is no reason why water systems should not be able to have some effect on the amounts of these materials in finished water. As I said before, the presence of THMs indicates a large number of other chemicals which are also there and even less understood relative to human risk.

Table 4. Uptake of chloroform for the adult human from air, water and food.

Source	Adult uptake, mg/year	Percent uptake
Maximum conditions		
Atmosphere	204	36
Water	343	61
Food supply	16	3
Total	563	100
Minimum conditions		
Atmosphere	0.41	13
Water	0.73	23
Food supply	2.00	64
Total	3.14	100
Maximum-water minimum-air		
Atmosphere	0.41	1
Water	343.00	97
Food supply	9.00	2
Total	352.41	100

Table 4 gives some numbers on the statement that water is normally the major contributor of these kinds of substances to our total exposure. Under some circumstances (minimum conditions) food intake might contribute more than the water. Under maximum conditions, water contributes more than the food although there can be a substantial portion from inhalation, too. But under conditions of moderate to heavy water exposure and typical atmosphere and typical food supply, the water is, by far, the major contributor. There are very few circumstances in environmental exposure to chemicals where one can pinpoint a route of exposure that so far outweighs the other sources as in this case.

Control of Trihalomethanes

There are many approaches that may be considered in the regulatory context on how to control the trihalomethanes:

- Use of a disinfectant (oxidant) that does not generate (or produces less) THMs in water.
- Treatment to reduce precursor concentrations prior to chlorination.
- Treatment to remove THMs after formation.

Many possible choices exist within each category. For example, alternate disinfectants or oxidants that might be considered include ozone, chlorine dioxide, and chloramines (combined chlorine). Precursor reduction processes include off-line raw water storage, aeration, improved coagulation, ion exchange resins, granular activated carbon (GAC), powdered activated carbon (PAC), and ozone enhanced biological activated carbon (BAC). THM reduction has also been achieved by merely moving the chlorine addition point to later stages in the conventional treatment process, and by substituting prechlorination with some other preoxidation process. THM removal processes include GAC, aeration or macroreticular resins.

Modifying disinfection practices is an area of particular interest. A number of other materials can perform as well as chlorine in the treatment of water as disinfectants or oxidants as a part of the total treatment process. But, of course, there are many questions about the toxicology and potential risks from those substances, too. Chlorine and the by-products of chlorination have been studied to a greater degree than ozone, chlorine dioxide and chloramines. These are also used in the United States, but also to a greater degree in other countries (ozone and ClO_2) and there is a long history for all these substances but little information on their toxicology.

The most important principle to remember is that in the course of these fine tuning activities, modifying disinfection practices, modifying dosages, or rearranging treatment processes, we cannot lose sight of the fact that the most significant health risk in drinking water is waterborne disease transmission. There is not only no excuse but there is no reason for taking any kind of action that would reduce the trihalomethanes, but, at some point along the way also increase the risks of waterborne disease. This is one of our great concerns, and, in writing the trihalomethane regulation, we were very cautious to take into consideration what we felt were the factors that would relate to the application of this regulation in all public water systems around the United States, particularly the small ones where there might be less of an opportunity or capacity for the water system to properly maintain biological quality as well as control chemical quality. So, in the risk-benefit equation, the risk-benefit balance, there should be no factor on the other side of the equation that allows for reduction of trihalomethanes and an increased probability of pathogenic bacteria or viruses being in the water.

Other Oxidants

The next questions to be asked are: what kinds of controls should exist on the use of not only chlorine or chlorine dioxide or other oxidants, or on the amounts of their residuals? At this point there is a recommended limit of 0.5 ppm on the sum of the oxychlorine by-products of chlorine dioxide (except for hypochlorite), and EPA said that, based upon new information, we would reconsider and determine whether an MCL would be appropriate. In the case of ozone, there are no particular restrictions, but we do point out that it is undoubtedly inadvisable to use ozone in many public water systems unless reasonably high quality water has first been produced. There is a very important place for ozone in water treatment, not as a substitute for chlorine, but rather as an integral part of a properly designed complete treatment process.

In the case of chloramines, are there excessive risks introduced by the wider use of chloramines rather than free chlorine? Is the toxicology of chloramines significant in the sense that these are more stable materials that are less likely to decompose early during ingestion and perhaps are going to interact with other systems in the body to a greater degree than some of the more active oxidants would? For example, does residual chlorine decompose in saliva, whereas chloramine might persist to or beyond the GI tract?

Conclusion

We must ask ourselves whether there are long-term risks to the population from consistent widespread exposure to any oxidants in water and their by-products. Are there particular safe levels when considering acute and subchronic and chronic toxicity apart from the carcinogenicity? Are there levels for chlorite, chlorate, chlorine dioxide, chloramines, or whatever in drinking water that should not be exceeded? Are there sensitive elements in the population that we must be particularly concerned about; those that would be more susceptible than the average person to these kinds of oxidant stresses? Toxicologists and epidemiologists must identify those people and find out what they can tolerate and help us answer these and the following questions. Does the systematic use of chlorine residuals in the United States introduce some additional increment of risk that would not have been there if residuals were not used? But, on the other hand, does the use of residuals add considerable benefits that far outweigh those potential risks? I mentioned the matter of the aerobic nature of the water from the existence of oxidants—are there particular benefits or particular risks b having an oxidized system rather than a reduce system? On the chemistry and toxicology, are ther great similarities between these disinfectants? (Ozon might not produce organochlorine compounds bu chlorine produces a vast array of oxidation product just like ozone does.) Are there significantly different products?

So, these are some of the questions that are bein examined. One of the top priorities in drinkin water health effects research is the matter o oxidants and disinfectants, and the Office of Drinking Water hopes that work will continue ver actively.

Environmental Health Perspectives
Vol. 46, pp. 7-12, 1982

Waterborne Outbreak Control: Which Disinfectant?

by Elmer W. Akin,* John C. Hoff† and Edwin C. Lippy*

Drinking water disinfection was shown to be an important public health measure around the turn of the century. In the United States, it was perhaps the single most important factor in controlling typhoid fever, a waterborne disease that was rampant throughout the world during the last century. It may also be assumed that disinfection was important in limiting the number of cases of other diseases known to be capable of waterborne transmission, i.e., cholera, amebiasis, shigellosis, salmonellosis, and hepatitis A.

Even though modern treatment has eliminated water as a major vehicle of infectious disease transmission, outbreaks still occur. In fact, the annual number has been increasing since 1966. Interruption in chlorination or failure to achieve adequate levels of chlorine residual is the most often identified deficiency of the involved water supplies. This finding indicates that waterborne microbial pathogens remain as a potential health threat and underscores the importance of disinfection.

From the outset, chlorination has been the drinking water disinfectant of choice in the country. Numerous studies have demonstrated its ability to inactivate bacterial, viral, and protozoal pathogens when applied under proper conditions. However, the finding that chlorinated organics that are potentially carcinogenic are formed has prompted an evaluation of alternative disinfectants. The viable alternatives to chlorine currently under consideration for widespread use are ozone, chlorine dioxide, and chloramines. In terms of biocidal efficiency, ozone is the most potent of the three. Chlorine dioxide is about the equivalent of free chlorine in the hypochlorous acid form but much more efficient than the hypochlorite form of free chlorine. The chloramines are weaker biocides than hypochlorite. Although this general order of ranking of efficiency holds for diverse types of microorganisms, quantitative comparisons vary with different microorganisms and experimental conditions.

Since the turn of the century, the benefits to public health from the disinfection of drinking water have been broadly recognized. The discovery that water could be a major vehicle of disease transmission preceded scientific verification of the germ theory of disease in the late 1800s. In 1854, John Snow made his classic deduction that a water supply was responsible for a severe cholera epidemic that was localized in a section of London, England. The outbreak that had caused 500 deaths was interrupted by removal of the handle on the Broad Street pump. A hint of the importance of water disinfection for disease control had actually been reported in 1835. *Human Health*, published in Philadelphia, stated that marsh water could be made potable by the addition of a small quantity of chlorine (*1*).

Concomitant with the recognition and acceptance of drinking water as a significant route of infectious disease transmission was the introduction of the disinfection process. In those early years, as now in this country, disinfection was synonymous with chlorination. In 1888, patents were issued on an

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electrolytic treatment process that generated chlorine from chloride of lime. The practice of chlorination of drinking water supplies spread throughout the major cities of the U.S. as the chlorine delivery process improved and experience revealed its microbial destructive power. It is interesting to note that the theory of chlorination chemistry that developed during the early 1900s indicated that nascent oxygen was responsible for the microbicidal action of the process. This theory, which also fostered interest in ozone as a disinfectant, was held until disproven in 1944 (*1*).

Waterborne Diseases

The most widely recognized achievement of drinking water treatment in the U.S. has been the dramatic reduction in typhoid fever which in large part is attributed to this single public health measure. The disease caused by an enteric bacterium was known to be waterborne with an estimated 40% of the cases attributed to this route of transmission. In 1900, the typhoid death rate in the U.S. had been 36 per 100,000 population, i.e., 25,000 deaths. As water treatment, including disinfection, became an increasingly common practice, the death rate decreased to 20 and 3 per 100,000 in 1910 and 1935, respectively. Perhaps Cincinnati's experience was typical. At the turn of the century, the city's water supply was the Ohio River with potability produced by only natural sedimentation to reduce settleable solids. The average annual typhoid fever illness rate during this time was almost 400 cases/100,000 persons. In 1907, the city added coagulation and rapid sand filtration treatment processes, and typhoid fever rates dropped precipitously. Chlorination, introduced in 1915, was followed by a second major reduction to a level less than one-tenth the rate reported for the year prior to introduction of water treatment (*2*).

Success in control of this disease by water treatment may better be illustrated by the decline in the reported cases of waterborne typhoid in the U.S. over the past 40 years (Fig. 1). In contrast to an annual average of 385 cases reported for the period 1940–45, no waterborne cases were reported during the 1976–79 period. A single typhoid outbreak resulting in 212 cases was responsible for increasing the annual average for 1971–75 from 2 to 44 cases. This outbreak resulted from the ingestion of contaminated ground water at a migrant work camp in Florida.

In addition to typhoid fever, other enteric pathogens have been associated with waterborne disease. The most widely recognized waterborne viral disease is hepatitis A. Mosley (*3*) compiled a list of 50 published reports worldwide of infectious hepatitis outbreaks attributed to contaminated drinking water. Epidemiological investigation has clearly shown this association even though disease manifestations normally do not appear until approximately 30 days after ingestion of contaminated water. Cholera and dysentery were rampant waterborne diseases during the 1800s but became less important in this century. Water disinfection most assuredly was important in their decline; however, the specific impact is not well documented.

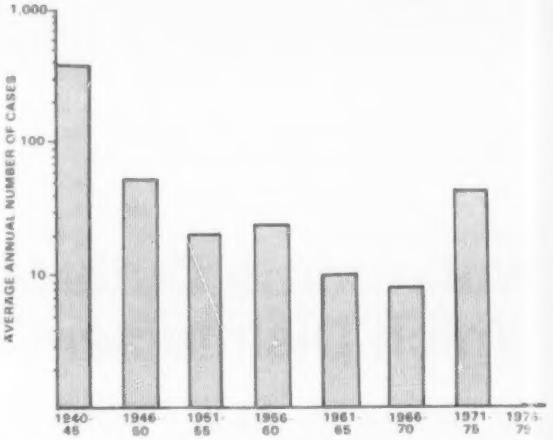

FIGURE 1. Average annual number of cases of typhoid fever in U.S. occurring in waterborne outbreaks from 1940 through 1979.

Waterborne Outbreaks

Although the number of cases of waterborne infectious disease has been greatly reduced since the adoption of a standardized treatment process for unprotected surface water, outbreaks still occur. Actually, the annual number of reported outbreaks has been increasing since 1966 (Fig. 2). In 1979, the latest year for which complete data are available, 43 outbreaks were reported that resulted in 7,500 cases of illness. Preliminary information for 1980 shows a further increase in the number of cases. Outbreaks are reported on a voluntary basis to the Centers for Disease Control by state health agencies. Generally, there is not a concentrated effort to document occurrence of outbreaks and significant under-reporting is suspected. EPA is attempting to determine the actual occurrence of waterborne outbreaks through the support of studies in three states. The objectives are to design, implement, and evaluate a comprehensive surveillance system.

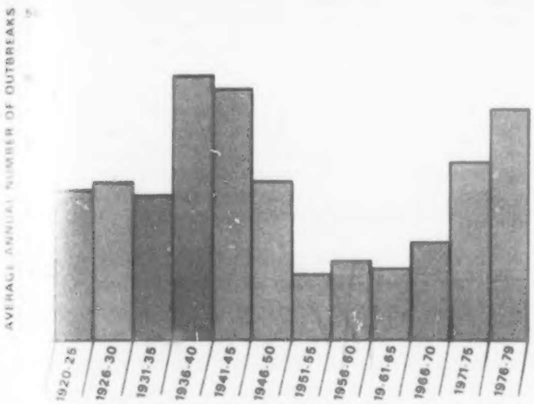

FIGURE 2. Average annual number of waterborne disease outbreaks occurring in U.S. from 1920 through 1979.

Investigations in one state uncovered seven outbreaks in the first 9 months of the study. In the previous year, only one outbreak had been reported through the normal surveillance procedures.

The most commonly occurring syndrome associated with waterborne illness is gastroenteritis that may be caused by a variety of microorganisms including viruses, bacteria, and protozoa. The frequency of each disease entity for outbreaks occurring between 1971 and 1977 is shown in Table 1. Acute gastrointestinal illness is a nonspecific category for which no known pathogens were identified. A group of viruses which have recently been observed by electron microscopy in the stools of outbreak victims is believed to be important in the etiology of this illness. Infectious hepatitis caused by hepatitis A virus is the most widely recognized waterborne virus illness. Epidemiological investigations have confirmed the waterborne transmission of this disease on numerous occasions. Since 1977, the protozoan *Giardia lamblia* has produced perhaps the largest number of waterborne disease cases and outbreaks. Its occurrence in "pristine" mountain streams contaminated by fecal waste of wild animals (especially beavers) has taken advantage of less vigorous water treatment procedures normally required for surface water sources (4). Inactivation of Giardia cysts requires more stringent control of the disinfection process than normally employed by many water utilities (5).

Conventional treatment, i.e., coagulation, sedimentation, filtration and chlorination, as it is commonly practiced in this country, coupled with an uncompromised distribution system, appears adequate to prevent waterborne infectious disease. However, the integrity of water systems is not assured and outbreaks are associated with breaches of these conditions. Table 2 indicates the causes of waterborne outbreaks in municipal and semipublic systems. Treatment deficiencies were important causes in both systems. Failure to maintain uninterrupted chlorination at an adequate chlorine concentration was found to be the single most important treatment deficiency (6). This was believed to be the cause of a recent gastroenteritis outbreak associated with a groundwater supply in a Texas community that affected approximately 8000 residents. The symptoms, i.e., rapid onset of diarrhea and abdominal cramps accompanied by headache and nausea, were typical of enteric microbial disease. However, no etiological agent has yet been identified. About 30 cases of hepatitis A were also associated with this outbreak (Lippy, unpublished data).

Water Disinfection

Although virus isolations from disinfected drinking water have been reported (7), most health authorities believe that chlorination, when prac-

Table 1. **Etiology of waterborne disease outbreaks in the U.S., 1971-1977.**[a]

Type of illness	Outbreaks, %	Cases of illness, %
Acute gastrointestinal illness	57	58
Chemical poisoning	12	3
Giardiasis	10	18
Shigellosis	9	14
Hepatitis A	8	1
Salmonellosis	2	3
Typhoid	2	<1
Enterotoxigenic *E. coli*	<1	3
	100	100

[a] Data of Craun (6).

Table 2. Waterborne disease outbreaks in the U.S., 1971-1977: type and deficiencies of water systems.[a]

Deficiency	Percent of outbreaks: Municipal systems	Percent of outbreaks: Semipublic systems
Untreated surface water[b]	14	9
Untreated ground water	10	43
Treatment deficiencies	28	40
Distribution deficiencies	40	2
Miscellaneous	8	6
	100	100

[a] Data from Craun (6).

[b] Includes giardiasis outbreaks in chlorinated, but not filtered, systems.

ticed under recommended conditions, is adequate for preventing the transmission of pathogens through drinking water. The current widespread use of free residual chlorine for disinfection of drinking water evolved as a result of developments in our knowledge of chlorination chemistry and the biocidal capabilities of various forms of chlorine. Early water chlorination practice was termed simple chlorination or marginal chlorination. It consisted of adding chlorine as a final step after filtration or the use of chlorine as the only treatment. In the early 1900s, observations indicating that bactericidal action continued even after free chlorine had disappeared, and the subsequent discovery that the combined chlorine products formed also had disinfecting capabilities led to the widespread use of chloramines. In part, this change was made because the use of chloramines eliminated the tastes and odors caused by the reactions of free chlorine with phenols and other organic compounds. Later, recognition of the superior biocidal capabilities of the hypochlorous acid form of free residual chlorine and development of the "breakpoint" concept resulted in changing to the current predominant practice of free residual chlorination.

During this period, a great variety of physical and chemical agents have been studied regarding their potential utility for water disinfection. Some, such as heat, are highly effective, but impractical because of cost. Others may be unsuitable because their effects are selective, e.g., quaternary ammonium compounds are effective bactericides, but ineffective viricides. Chemical oxidants are the most widely used and potentially usable agents. These include the halogens such as bromine, iodine, and chlorine in various forms, metal salts such as ferrate and permanganate, and peroxides such as ozone and hydrogen peroxide. Of these, only ozone, chlorine dioxide and chloramines are currently considered to be immediately viable alternatives to free residual chlorine for use as primary disinfectants for potable water. This consideration is based mainly on the fact that these agents have been used successfully in actual treatment practice. The use of chloramines in some treatment systems has continued, with apparent success, in spite of laboratory data indicating that they are relatively poor disinfectants.

Comparative Biocidal Efficiency

The assessment of the biocidal efficiency of disinfectants is based mainly on the results of laboratory experiments conducted under controlled conditions. Some of the problems associated with such assessments were described by Morris (*8*) as follows:

> "Although the bases for the quantitative expression of the effectiveness of germicidal agents have been known for more than 60 years, there has been relatively little application to the systematic tabulation of the relative potencies of disinfectants. Only a very small fraction of the total published literature on germicidal action is sufficiently complete or in form suitable for satisfactory quantitative analysis. Moreover, there is no consensus on which method of tabulation is most convenient. Probably the most common technique is to list the concentrations required to give a fixed percentage of kill with a given time of contact, but there is no unanimity with regard to either the percentage or the time."

Inactivation of microorganisms by chemical disinfectants can be considered as a first-order chemical reaction in which the rate of inactivation is dependent on the disinfectant type and species, the disinfectant concentration, and the microorganisms being inactivated. Although actual data often show deviation from first-order kinetics, it is a useful concept. Using data in this way, comparative biocidal efficiency can be expressed as the relative concentration of various disinfectants required for equivalent disinfection rates or as the relative inactivation rates produced by equivalent concentrations of different agents. Data from laboratory experiments conducted at constant disinfectant levels and with pure cultures of the organism are shown in Figures 3 and 4. The data shown are composites of results from studies conducted over a period of years in one laboratory using consistent experimental methods and microorganism strains (*9*).

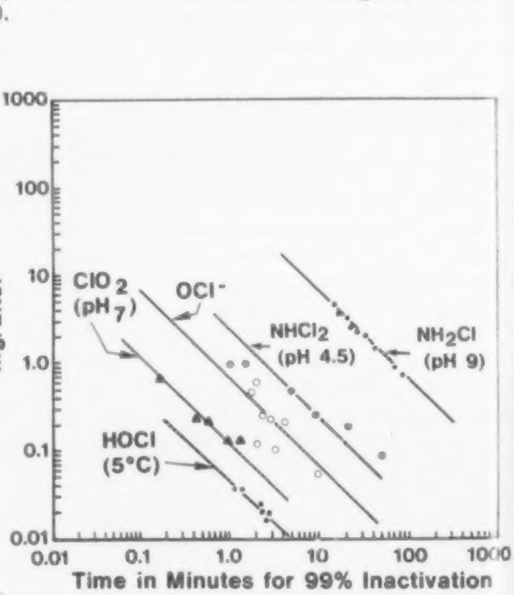

FIGURE 3. Inactivation of *E. coli* (ATCC 11229) by free and combined chlorine species and chlorine dioxide at 15°C (*9, 13*).

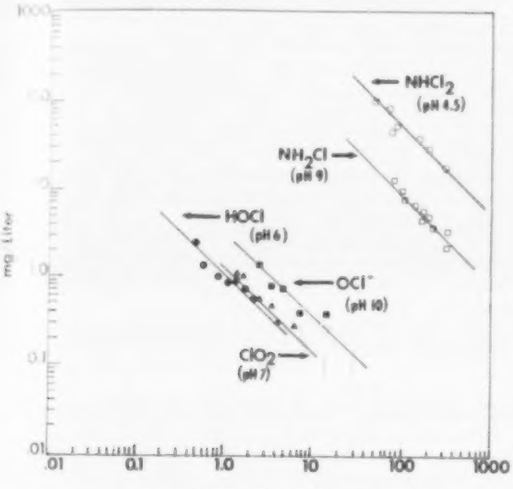

FIGURE 4. Inactivation of poliovirus 1 by free and combined chlorine species and chlorine dioxide at 15°C (*9, 13*).

Figure 3 shows the times and concentrations of several disinfectants required to cause inactivation of 99% of a population of *Escherichia coli*. Free residual chlorine in the form of hypochlorous acid is somewhat more effective than chlorine dioxide, but in its other form, hypochlorite ion, free residual chlorine is less effective than chlorine dioxide. Dichloramine is somewhat less effective than hypochlorite ion and monochloramine is the least effective. The overall pattern shown by results of studies of poliovirus 1 inactivation (Fig. 4) is similar. Note that all of the curves are further to the right, indicating the generally higher resistance pattern of viruses than *E. coli* to the disinfectants. Hypochlorous acid and chlorine dioxide are similar in efficiency with hypochlorite ion somewhat less effective. Both chloramine species are much less effective, but their order of efficiency is reversed from that shown for *E. coli*. Studies similar to these with ozone are difficult because the instability and extremely rapid inactivation rates shown by this disinfectant make it difficult to obtain reproducible results. Studies that have been done indicate that ozone is perhaps two to three orders of magnitude more efficient than free residual chlorine (*10, 11*). The overall efficiency rankings indicated in Figures 3 and 4 are consistent in general with the disinfection literature for all microorganisms including bacteria, viruses and protozoan cysts. However, precise quantitative ranking of the disinfectants with regard to the degree of difference in efficiency is not possible.

Water pH has important effects on the efficiency of free residual chlorine and chlorine dioxide. In the case of free residual chlorine, increasing the pH from 6 to 10 alters the disinfectant species present from a very efficient nonionized chemical species to a much less efficient ionized species. In contrast, the efficiency of chlorine dioxide increases over a similar pH range. Both *E. coli* (*12*) and poliovirus 1 (*13*) are inactivated more rapidly at pH 9 than at pH 7 by chlorine dioxide. In this case it appears to be a change in the sensitivity of the organisms since chlorine dioxide remains the same chemically over this pH range. Further evidence that different microorganisms are affected differently by disinfectants is shown in Table 3 (*14*). This group of six enteroviruses show widely differing patterns of resistance to both hypochlorous acid and hypochlorite ion. Relative differences in resistance to hypochlorous acid and hypochlorite ion range from 5-fold to 192-fold for different enteroviruses. In the same study, they showed that the presence of an inorganic salt (KCl) can significantly influence the rate of inactivation of viruses by both free chlorine species. Others have confirmed this observation and have shown that similar effects are produced by NaCl and CsCl (*15–17*). More recently, Haas and Zapatkin have shown that *E. coli* is similarly affected (*18*).

Choosing a Disinfectant

The important considerations in choosing a disinfectant appear to be technical rather than economic in nature. Cost comparisons have not been presented here. However, Clark (*2*) has determined that while operating cost of using the disinfectants under discussion may vary 3- to 4-fold, when considered in terms of cost-benefit ratios, all three show a highly positive net benefit.

An important consideration in choosing a disinfectant for use in water treatment is the stability of the disinfectant species. In general, degree of

Table 3. Comparison of virus inactivation by free residual chlorine at pH 6.0 and 10.0, and 5.0°C ± 0.2°C.[a]

	Time for 99% inactivation, min		
Virus strain	pH 6.0	pH 10.0	Ratio[b]
Coxsackie A9 (Griggs)	0.3	1.5	5
Echo 2 (Farouk)	0.5	96.0	192
Polio 2 (Lansing)	1.2	64.0	53
Echo 5 (Noyce)	1.3	27.0	21
Polio 1 (Mahoney)	2.1	21.0	10
Coxsackie B5 (Faulkner)	3.4	66.0	19

[a]Data of Engelbrecht et al. (*14*).
[b]Time required at pH 10.0/time required at pH 6.0.

Table 4. Summary of disinfectant characteristics relating to biocidal efficiency.

Disinfectant	Rank[b] Biocidal efficiency	Rank[b] Stability	pH effects on efficiency (pH range 6–9)
Ozone	1	4	Little effect
Chlorine dioxide[c]	2	2	pH increase is beneficial
Free chlorine[c]	3	3	pH increase is detrimental
Chloramines	4	1	Little effect

[a] Data of Hoff and Geldreich (*19*).

[b] 1 = best, 4 = worst.

[c] Ranking influenced by pH

stability correlates inversely with biocidal efficiency, the more stable the disinfectant, the less efficient. Although chlorine dioxide is similar in disinfection capability to hypochlorous acid, it is considered to be more stable because it does not react with ammonia which is frequently present in raw waters. However, disinfectant stability can also be considered beneficial because stable disinfectants such as chloramines, although less efficient, may be very effective because of their prolonged persistence. An overall view of some of the characteristics of ozone, chlorine dioxide, and free and combined chlorine is shown in Table 4. It is clear that none of the agents is optimal in all respects. The current concern regarding disinfection and the production of potentially toxic substances, the subject of this symposium, further complicates this picture. Perhaps the only firm conclusion that can currently be drawn concerning drinking water disinfection is its absolute requirement, by some means, in the treatment process if the transmission of waterborne infectious disease is to be prevented.

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Environmental Health Perspectives
Vol. 46, pp. 13-17, 1982

Toxicological Effects of Chlorine Dioxide, Chlorite and Chlorate

by **Daniel Couri,* Mohamed S. Abdel-Rahman† and Richard J. Bull‡**

Review of the available literature obtained from both acute and chronic experiments utilizing rats, mice and chickens treated with ClO_2, ClO_2^- and ClO_3^- in drinking water has demonstrated alterations in hematologic parameters in all species tested. The effects were usually dose related and marked changes occurred only at the higher dosages (up to 1000 mg/l.). In chronic studies, rats have been given ClO_2 at doses of up to 1000 mg/l., and $NaClO_2$ or $NaClO_3$ at up to 100 mg/l., in their drinking water for one year. Treatment groups receiving ClO_2, ClO_2^- or ClO_3^- showed alterations in erythrocyte morphology and osmotic fragility; at higher dosages mild hemolytic anemia occurred. An examination of blood glutathione content and RBC enzymes involving glutathione formation showed a dose-related diminution of glutathione in chlorine compound treated groups. The higher oxidative capacity of the chlorine compounds resulting in the decreased erythrocytic glutathione might well be the principal biochemical event leading to the other hematological alterations.

More recent data show that ClO_2, ClO_2^- and ClO_3^- alter the incorporation of ^{3}H-thymidine into the nuclei of various organs of the rat. These data suggest the possibility of increased turnover cells of the gastrointestinal mucosa and inhibited DNA synthesis in several organs. In the latter category, most concern revolves around whether or not the apparent depression of DNA synthesis in the testes is associated with depressed spermatogenesis and reproductive toxicity in the male rat.

Introduction

Institution of disinfection to drinking water treatent has been one of the key successes of public alth policy. In the United States almost complete ependence has been placed on chlorine for primary sinfection. In recent years, however, it has become ear that treatment of drinking water with chlone results in the formation of trihalomethanes (*1*, . One of the trihalomethanes, chloroform, has een shown to be carcinogenic in mice and rats (*3*). These observations stimulated a search for a means to minimize the formation of these products. One of the most attractive alternatives is to substitute disinfection methods that do not promote the formation of chlorinated by-products.

Chlorine dioxide is a very attractive alternative to chlorine as a disinfectant. For all intents and purposes its disinfectant properties are equivalent to or perhaps exceed those of chlorine (*4*). Chlorine dioxide does not react with phenol to produce the same taste and odor problems that result from chlorine treatment (*5*). It has the added advantage that formation of trihalomethanes does not occur (*6*, *7*).

Prior to the recent consideration of chlorine dioxide as a primary disinfectant for potable water, very little information existed concerning its toxi-

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cology. The use of chlorine dioxide for this purpose also requires consideration of by-products of its use that will occur in the consumed product, chlorite (ClO_2^-) and chlorate (ClO_3^-). Chlorite occurs both as a result of being the first reduction product in oxidative reactions in which ClO_2 participates and because ClO_2 is often generated by acidifying a sodium chlorite feed solution. Chlorate on the other hand arises primarily by a disproportionation reaction of ClO_2 that is catalyzed by ultraviolet light (*8*), giving rise to one molecule of ClO_2^- and one molecule of ClO_3^- per two molecules of ClO_2.

As a result of the interest in ClO_2 as a primary disinfectant, a number of studies have been initiated to investigate the toxicity associated with ClO_2, ClO_2^- and ClO_3^-. This paper reviews the literature which has resulted from these investigations and attempts to deal with the mechanisms involved with the cellular damage induced by these agents.

Hematologic Effects

Rats, mice and chickens have been treated with ClO_2, ClO_2^- and/or ClO_3^- in drinking water at concentrations ranging from 1 to as high as 1000 mg/l. A relatively consistent picture has been observed across species. In the rat, Heffernan et al. (*9*) demonstrated that chlorite concentrations of 100 mg/l. and above resulted in decreased red blood cell counts, hemoglobin concentrations and packed cell volume at 30 and 60 days of exposure. These effects tended to be reversed by 90 days of exposure despite continued treatment with sodium chlorite. These data were extended to the mouse by Moore and Calabrese (*10*) with very similar results. Abdel-Rahman et al. (*11*) studied the effects of ClO_2 and ClO_3^- as well as ClO_2^- in both the rat and chicken for periods of up to 9 months. Signs of anemia observed in shorter term exposures of 30 to 60 days (*12*) had substantially disappeared in this interval with ClO_2 and ClO_2^-. However, the results obtained with ClO_3^- at 9 months indicated 13 and 28% decreases in RBC at 10 and 100 mg/l. This represented a considerably greater effect than had been observed at shorter time intervals.

Accompanying the relatively small decreases in RBC count is subclinical evidence of increased red cell destruction. This was first reported by Heffernan et al. (*9*) in the cat, where significant decreases in the half-life of erythrocytes were observed at concentrations of ClO_2^- of 100 mg/l. of drinking water and above. No effect was observed at 10 mg ClO_2^-/l. Other investigators have provided evidence of distortions in erythrocyte morphology in mice (*10*) as well as the rat and chicken (*12*). These changes were quite marked at concentrations of 100 mg ClO_2^-/l. ranging from mildly crenated erythrocytes which maintained their discoid shape to crenated spheres (echinocytes). With ClO_3^- treatment, codocytes (Mexican hat-shaped) were also observed in the rat. In the chicken, the characteristic abnormality was the dacrocyte, which was evident primarily with ClO_2 treatments of 100 and 1000 mg/l. Early studies had somewhat paradoxically indicated that ClO_2, ClO_2^- and ClO_3^- decreased osmotic fragility of erythrocytes isolated from treated animals (*12*). Subsequent experiments have shown this effect is most likely due to the oxidation of thiol groups resulting in the precipitation of hemoglobin to give falsely low indications of hemolysis (*13*). This is also consistent with the *in vitro* observations of Heffernan et al. (*14*) that a complete loss of measurable pigment (both hemoglobin and methemoglobin) occurred at high concentrations of ClO_2^-.

Biochemical Effects

Early reports had documented the ability of ClO_2^- to oxidize hemoglobin to methemoglobin by high intraperitoneal doses (320 mg/kg) (*15*).

Heffernan et al. (*9*) confirmed these observations using intraperitoneal doses in the rat and bolus oral doses of ClO_2^- in the cat. However, in studies where ClO_2, ClO_2^- or ClO_3^- was included in the drinking water for up to several months, no significant increases in methemoglobin concentrations resulted with doses as high as 1000 mg/l. in the rat, mouse or chicken (*9*, *10*, *12*).

Therefore, although a real hazard with large bolus doses, methemoglobinemia appears to be an unlikely hazard at concentrations of ClO_2, ClO_2^- and ClO_3^- which might be anticipated to be associated with drinking water treatment.

Heffernan et al. (*9*) reported that ClO_2^- incubated with RBC *in vitro* depleted these cells of glutathione and increased the level of hydrogen peroxide produced as measured by catalase complex 1 formation. These *in vitro* studies clearly demonstrated that virtually complete loss of glutathione occurred before there was significant accumulation of methemoglobin. Additionally, these studies demonstrated a biphasic consumption of added ClO_2^-, one phase of which was associated with hydrogen peroxide generation and glutathione depletion and the other with hemoglobin oxidation. The changes in erythrocyte morphology by scanning electron microscopy *in vitro* paralleled exactly the progression observed with ClO_2 treatment in subsequent *in vivo* experiments (*10*, *12*).

From these data it was clear that ClO_2^- possesses the types of activity associated with chemicals that produce oxidative hemolysis *in vivo* (*16–18*).

Red blood cell glutathione concentrations have proven to be the most sensitive measure of ClO_2, ClO_2^- and ClO_3^- induced hematological effects *in vivo* as well. Heffernan et al. (*9*) observed significant depletions of glutathione and increases of 2,3-diphosphoglycerate in the red cells of rats exposed to as little as 50 mg of ClO_2^-/l. of drinking water. Unlike the effects on RBC counts and hemoglobin concentrations, tolerance does not develop to ClO_2^--induced depletion of glutathione. Abdel-Rahman et al. (*11*, *12*), confirmed this result with ClO_2^- and extended the observations to ClO_2 and ClO_3^-. Like ClO_2^-, ClO_3^- produced stable depression of red cell glutathione concentrations with continued treatment at 10 and 100 mg/l. On the other hand, depression of red cell glutathione levels by ClO_2 tends to disappear with continued exposure (*11*, *13*).

The recovery or nonrecovery of glutathione levels with continued treatment *in vivo* appeared at least partially dependent upon a complex set of interactions with redox systems operating in the rat erythrocyte (*19*). The reversibility of the ClO_2 effect appears to result from increased levels of glutathione reductase activity in the red cell following 6 months of treatment. In the case of ClO_3^- there appears to be no such adaptive increase in this enzyme level. With ClO_2^- the glutathione reductase levels do increase to levels comparable to those observed with ClO_2. However, this adaptive change appears to be offset by significantly depressed levels of catalase in the red cell with ClO_2^- treatment.

The importance of the changes in enzyme levels in the adaptation to chronic ClO_2 exposure has been indirectly supported by the data of Heffernan et al. (*9*, *14*). First, the addition of glucose to red cells treated with ClO_2^- *in vitro* partially protects against depletion of glutathione. Glutathione peroxidase is the principal means of disposing of hydrogen peroxide in the red cell (*17*). Glucose serves as the carbon source which donates the reducing equivalents necessary for the reduction of oxidized glutathione (*17*, *20*).

Secondly, *in vivo* treatment with ClO_2^- greatly enhances the formation of ClO_2^--induced formation of hydrogen peroxide *in vitro*. Clearly *in vivo* treatment with ClO_2^- reduces the ability of the red cell to dispose of hydrogen peroxide. Although the animal is not seriously ill at levels of ClO_2, ClO_2^- or ClO_3^- considerably exceeding those contemplated in drinking water treatment, the body's ability to adapt to oxidant stress is compromised at relatively low concentrations of these chemicals.

These considerations focus attention on populations known to be sensitive to oxidants, for example, those deficient in glucose-6-phosphate dehydrogenase. Such individuals are known to be considerably more sensitive to oxidant stress than normal humans. As will be discussed in other papers in this symposium, erythrocytes isolated from such individuals are three to four times as susceptible to ClO_2^--induced damage than normal human red cells (*21*). In this regard, Michael et al. (*22*) conducted a human study in a small town which utilized ClO_2 in combination with chlorine as a primary disinfectant during the summer months. Concentrations of ClO_2, ClO_2^- and ClO_3^- were not closely controlled but the total of their concentrations averaged 5–7 mg/l. In this situation normal humans were not apparently affected. However, the only individual in the community deficient in glucose-6-phosphate dehydrogenase experienced a substantial decrease in hemoglobin levels and hematocrit. With only one case it is difficult to conclusively ascribe this change to ClO_2 or its by-products, but it does suggest reason for concern about sensitive populations.

Evidence of Damage to Other Tissues

Research examining nonhematologic effects of ClO_2, ClO_2^- and ClO_3^- is sparse. Distribution and clearance of ^{36}Cl derived from ClO_2, ClO_2^- or ClO_3^- is widespread throughout all organs including bone marrow (*12*, *23*, *24*).

There is some evidence of macromolecular binding in the liver. These findings raised the question of whether or not cellular types in addition to the red cell are similarly targeted by these chemicals.

The gastrointestinal mucosa is the obvious first potential target of residual disinfectant species. Abdel-Rahman et al. (*11*) examined the incorporation of ^3H-thymidine into nuclei of cells of the intestinal mucosa of the rat as a measure of the turnover of these cells. At levels of 10 mg/l. of ClO_2 and ClO_2^- in drinking water for 3 months, there were significant increases in ^3H-thymidine incorporation, implying an increased turnover of intestinal epithelium. No effect was observed with equivalent concentrations of ClO_3^-. Unfortunately, lower levels of exposure to ClO_2 and ClO_2^- were not examined.

Examination of other organs revealed that ClO_2 treatment inhibited ^3H-thymidine incorporation into nuclei of cells in the kidney and testes of the rat. ClO_2^- produced similar effects in the testes and markedly inhibited ^3H-thymidine incorporation in the liver at 10 and 100 mg/l. ClO_3^- produced significant effects only in the testes. At 100 mg/l, ClO_2^- also inhibited the incorporation of ^{3}H-thymidine into the gastrointestinal mucosa and ClO_2 produced a smaller increase than had been observed with 10 mg/l. These data suggest that at high doses in the intestinal mucosa and at lower doses in testes and liver, ClO_2 and/or its by-products is interfering with DNA synthesis. The significance of these results is presently difficult to determine. But since a large portion of the DNA synthesis ongoing in testes is associated with spermatogenesis, these results cannot be lightly dismissed. Consequently, it is essential that this work be followed up with studies of the reproductive toxicity of these chemicals and be extended to lower doses.

Conclusions

It is clear from this brief review that substantial questions remain to be answered concerning the safety of ClO_2 as a primary disinfectant of drinking water. Although ClO_2, ClO_2^- and ClO_3^- have been used extensively in drinking water treatment, actual human exposure has been limited in the past because the concentrations proposed for taste and odor control are much lower than required for primary disinfection. Europeans have used ClO_2 as a primary disinfectant, but again levels which have been used are quite low because disinfectants are employed after much of the organic material has been removed from the water by granular activated carbon, a practice not common in the U.S. Consequently, only in certain very limited circumstances have epidemiological evaluations been possible and appropriate to the use of ClO_2 as a primary disinfectant. Consequently, it appears that further experimental work will be necessary to more clearly identify and quantify the hazards involved with its use.

Three major areas of concern over the use of ClO_2 as a disinfectant have been identified by research performed to date. First, there is a need to study human populations which display higher levels of sensitivity to oxidant chemicals. Second, efforts must be made to determine the significance of increased turnover of the epithelium of the gastrointestinal tracts at low doses of ClO_2 and ClO_2^-. This tissue normally turns over quite rapidly. On the other hand, cellular damage followed by regeneration has been associated with increased susceptibility to chemical carcinogens in other organs such as the liver (*25*). Third, and perhaps most important, is the question of potential reproductive effects of ClO_2 and its by-products implied, but not established, by the reduced incorporation of ^{3}H-thymidine into testicular DNA. The fact that this effect occurred at quite low doses of ClO_2, ClO_2^- and ClO_3^- raises the level of concern considerably. It should be noted that similar effects were not observed with HOCl (*11*).

In summary, there is little conclusive evidence to prevent the use of ClO_2 as a disinfectant at present. On the other hand, there are scattered pieces of indirect evidence that would advise caution about instituting the use of ClO_2 as a primary disinfectant in place of chlorine. This is complicated by the fact that the total concentration of ClO_2, ClO_2^- and ClO_3^- might reach several parts per million in the absence of removing background organic material to be an effective disinfectant. It is obvious that this question can only be finally resolved through further research.

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Environmental Health Perspectives
Vol. 46, pp. 19-23, 1982

Metabolism and Pharmacokinetics of Alternate Drinking Water Disinfectants

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The chlorination of surface waters is known to elevate trihalomethanes; consequently, chlorine dioxide (ClO_2) is being considered as an alternative disinfectant. The primary products resulting from ClO_2 disinfection of waters are chlorites (ClO_2^-) and chlorates (ClO_3^-). Studies in rats revealed that ClO_2 is converted to chloride (Cl^-), ClO_2^- and ClO_3^-. ClO_2^- and ClO_3^- are excreted as Cl^-, ClO_2^- and Cl^-, ClO_2^-, ClO_3^-, respectively. Radioactivity was rapidly absorbed from the gastrointestinal tract following the administration of $^{36}ClO_2$ orally, and the half-life for the elimination of ^{36}Cl from the rat was 44 hr, corresponding to a rate constant of 0.016/hr. After 72 hr, radioactivity was highest in plasma, followed by kidney, lung, and stomach. ^{36}Cl in plasma reached a peak at 2 hr and 1 hr after oral administration of $^{36}ClO_2^-$ and $^{36}ClO_3^-$, respectively. ^{36}Cl excretion was greatest 24 hr after the administration of $^{36}ClO_3^-$, but in the case of $^{36}ClO_2^-$, the excretion probably represented saturation of the biotransformation and excretion pathway. A low activity in packed cells compared to plasma was detected in chlorate ingestion, rather than an even distribution in chlorite treatment. Chloroform determinations in rat blood after one year indicated that chloroform was significantly higher than control in the 100 and 1000 mg/l. ClO_2 groups. However, no significant values were observed in the 1 or 10 mg/l. ClO_2 and ClO_2 metabolites group. ClO_2 and its metabolites are eliminated from the body more rapidly than chlorine, and they do not appear to increase trihalomethane concentrations at low dosages.

Introduction

Chlorination is, and always has been, the most widely used method for disinfection of public water supplies. Concern over its possible adverse effects and those of its by-products on humans has increased considerably in recent years. The formation of trihalomethanes such as chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), dibromochloromethane ($CHBr_2Cl$) and bromoform ($CHBr_3$) during the chlorination process has become evident (*1*, *2*).

The United States Environmental Protection Agency conducted a comprehensive survey of 80 water supplies in the U.S. for halogenated organics (*3*). It was found that the four trihalomethanes—$CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$, $CHBr_3$—are widespread in chlorinated drinking water in the U.S. and result from chlorination, since these substances were not found in raw waters. The trihalomethanes were not a result of impurities in the chlorine being used but were a result of the reaction of chlorine with precursor. Morris (*4*) made the point that the number of trihalomethane precursors is extensive and includes ethanol, acetaldehyde and the methyl ketones. Rook (*1*) and Morris (*4*) indicated that the bromide ion present in a natural water is rapidly oxidized by aqueous chlorine to hypobromous acid (HOBr), and that this acid reacts with precursor to account for the formation of the brominated species.

Since these trihalomethanes may be a health hazard, chlorine dioxide (ClO_2) is one of the alternative disinfectants being considered because it prevents the formation of trihalomethanes (*5*, *6*). ClO_2 is a more effective oxidizing agent than chlorine and greatly improves the taste of water by avoiding the unpleasant taste associated with chlorination of

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water (*7*). The unpleasant taste of chlorine in water is caused partially by the build-up of chlorinated phenols.

Miltner (*6*) has reported that the primary products resulting from ClO_2 disinfection of surface waters include chlorites (ClO_2^-) and chlorates (ClO_3^-), which appear in concentrations of 50% and 30% of ClO_2 demand, respectively.

Toxicity studies demonstrated that rats drinking ClO_2, ClO_2^- and ClO_3^- (Cl compounds) daily for 9 months exhibited depressed red blood cell counts, hemoglobin concentration and packed cell volumes. Also, these Cl compounds in drinking water for three months inhibited the incorporation of ^3H-thymidine into nuclei of rat testes (*8*).

The studies described in this report were conducted to provide information on metabolism and pharmacokinetics of chlorine, chlorine dioxide, chlorite and chlorate in the rat. Also, the effect of ClO_2 in drinking water on the formation of $CHCl_3$ in rat blood and various organs was studied.

Methods

Synthesis of ^{36}Cl Compounds

$H^{36}Cl$ was obtained from New England Nuclear. This material, with a radionuclidic purity of > 99% was used as the source of ^{36}Cl. The potassium chlorate ($K^{36}ClO_3$) was synthesized according to the method of Abdel-Rahman et al. (*9*). The generation of $^{36}ClO_2$ from $K^{36}ClO_3$ was accomplished by the reaction (1). $^{36}Cl_2$ was formed by the reaction (2).

$$2K^{36}ClO_3 + (COOH)_2 + 2H_2SO_4 \rightarrow 2^{36}ClO_2 + 2KHSO_4 + 2CO_2 + 2H_2O \quad (1)$$

$$KMnO_4 + 16H^{36}Cl \rightarrow 2K^{36}Cl + Mn^{36}Cl_2 + 5^{36}Cl_2 + 8H_2O \quad (2)$$

The purity of the synthesized $K^{36}ClO_3$, which is an essential intermediate step to the production of ClO_2, was 95%, as determined by fast neutron activation analysis.

Kinetics of Chlorine Dioxide, Chlorite, Chlorate and Chlorine in the Rat

Four groups of four male Sprague-Dawley rats (230 ± 20 g) each were used in these experiments. ^{36}Cl compounds were administered by gavage as follows: 3 ml of 100 mg/l. $^{36}ClO_2$ (0.7 μCi) was administered to each rat, producing a dose of 1.5 mg/kg body weight; 3 ml of 10 mg/l $^{36}ClO_2^-$ (0.17 μCi) was administered to each rat, producing a dose of 0.15 mg/kg body weight; 3 ml of 5 mg/l. $^{36}ClO_3^-$ (0.85 μCi) was administered to each rat, producing a dose of 0.065 mg/kg body weight; 3 ml of 250 mg/l. $HO^{36}Cl$ (0.45 μCi) was administered to each rat, producing a dose of 3.26 mg/kg body weight.

Heparinized blood samples were collected at 5, 10, 20, 30 and 60 min, and 2, 4, 8, 24 and 48 hr by orbital sinus puncture. The blood was centrifuged at 1000*g* for 15 min to separate the red blood cells from the plasma. Then the radioactivity was counted in a liquid scintillation counter (Beckman LS 7500), and the rate constant and $T\frac{1}{2}$ of absorption and elimination from plasma were calculated for each compound.

Distribution of Cl Compounds in the Rat

After 72 hr following the administration of ^{36}Cl compounds as described previously, rats were killed by decapitation, and blood was collected in heparinized tubes. Tissue specimens of stomach, testes, lung, kidney, duodenum, ileum, spleen, liver, bone marrow, carcass and skin were prepared for the determination of ^{36}Cl content by liquid scintillation spectrometry as follows: Whole blood and packed cells were prepared according to the method of Mahin et al. (*10*). Samples of wet tissue weighing up to 200 mg and samples of bone marrow up to 50 mg were placed in individual glass LSC vials with Teflon or polyethylene-lined caps. Then 2 ml Protosol was added to each vial, which was capped tightly to prevent loss of solvent. Samples were solubilized by heating overnight at 50°C, then cooled at room temperature; afterwards 0.2 ml of 30% H_2O_2 was added for decolorization. The samples were heated at 50°C for 30 min, then cooled, and 15 ml of Aquasol-2 was added.

Sample quench effects were corrected by using the method of channel ratios, and efficiency was greater than 80%.

Metabolism and Excretion Studies

Four groups of four male Sprague-Dawley rats/group were used in these studies. Each group was administered orally the same concentrations of ^{36}Cl compounds described above. Animals were housed in modified Roth all-glass metabolism chambers for the collection of expired air and fecal and urine samples. The radioactivity of the total ^{36}Cl compounds was measured. The analysis of radioactive metabolites (Cl^-, ClO_2^-, ClO_3^-) was performed as described in a previous report, (*9*), and measured as a percentage of the initial dose.

Effect of Chlorine Dioxide, Chlorite and Chlorate on the Formation of Chloroform in Rat Blood and Organs

Male Sprague-Dawley rats (50–170 g) were used in this experiment. The animals were housed in a controlled environment (temperature, 22°C; humidity 50%) with a 12 hr light/dark cycle beginning at 6:00 A.M. Food was available *ad libitum*. Groups of rats were allowed to drink doubly distilled water containing 10 or 100 mg/l. ClO_2, ClO_2^- or ClO_3^- for 20 hr/day, 7 days/week for 12 months. ClO_2 was generated daily and water concentrations determined by the DPD (diethyl-*p*–phenylenediamine) method of Palin (*11*).

After one year, rats were killed by decapitation and blood collected in heparinized tubes. Tissue samples of liver, kidney, spleen, brain, and testes were homogenized in isotonic sucrose medium. Tissue homogenate or blood sample (3 ml) was extracted by 5 ml pentane. The pentane layer was then separated by centrifugation at 1000*g* for 5 min. A gas chromatograph equipped with an electron capture detector was used for the quantitation of chloroform in each sample, with bromodichloromethane being used as the internal standard.

Results

Kinetics of Cl Compounds in the Rat

The rate constant and $T_{1/2}$ for absorption and elimination from plasma of Cl compounds are shown in Table 1. The rate constant for absorption was highest for $^{36}ClO_2$ (3.77 ± 0.24/hr), followed by $^{26}ClO_3^-$, $^{36}ClO_2^-$, and lowest for $HO^{36}Cl$ (0.157 ± 0.001/hr). The $T_{1/2}$ for absorption, which is inversely proportional to the rate constant, was longest for $HO^{36}Cl$ (4.42 ± 1.31 hr), followed by $^{36}ClO_2^-$, $^{36}ClO_3^-$, and was shortest for $^{36}ClO_2$ (0.18 ± 0.01 hr). For elimination from plasma, the rate constant was highest for $^{36}ClO_2^-$ (0.02 ± 0.002/hr), followed by $^{36}ClO_3^-$, $^{36}ClO_2$ and $HO^{36}Cl$ (0.009 ± 0.001/hr). The $T_{1/2}$ elimination from plasma was longest for $HO^{36}Cl$ (77.0 ± 8.8 hr), followed by $^{36}ClO_2$, $^{36}ClO_3^-$, and $^{36}ClO_2^-$ (35.2 ± 3.0 hr).

Distribution of Cl Compounds in the Rat

The distribution of ^{36}Cl compounds after 72 hr from the time of oral administration is shown in Table 2. Values are expressed as a percentage of

Table 1. Kinetic constants of chlorine dioxide, chlorite, chlorate and chlorine in the rat.[a]

	Absorption, hr^{-1}		Elimination, hr^{-1}	
Treatment	Rate constant	$T_{1/2}$	Rate constant	$T_{1/2}$
$^{36}ClO_2$ (100 mg/l.)	3.77 ± 0.24	0.18 ± 0.01	0.0158 ± 0.0008	43.9 ± 2.3
$^{36}ClO_2$ (10 mg/l.)	0.198 ± 0.06	3.50 ± 1.06	0.0197 ± 0.0017	35.2 ± 3.0
$^{36}ClO_3$ (5 mg/l.)	0.399 ± 0.151	1.74 ± 0.66	0.019 ± 0.003	36.7 ± 5.8
$HO^{36}Cl$ (250 mg/l.)	0.157 ± 0.001	4.42 ± 1.31	0.009 ± 0.001	77.0 ± 8.8

[a] Values were calcuiated from the time course of the elimination from rat plasma; *n* = 4 rats per treatment.

Table 2. Chlorine dioxide, chlorite, chorate and chlorine distribution in the rat.

	Percentage of initial dose[a]			
Tissue	$^{36}ClO_2$ (100 mg/l.)	$^{36}ClO^-_2$ (10 mg/l.)	$^{36}ClO^-_3$ (5 mg/l.)	$HO^{36}Cl$ (250 mg/l.)
Plasma	0.72 ± 0.02	0.55 ± 0.038	0.68 ± 0.09	0.77 ± 0.04
Packed cells	—[b]	0.63 ± 0.11	0.23 ± 0.02	—
Whole blood	—	0.64 ± 0.01	0.57 ± 0.05	—
Kidney	0.81 ± 0.15	0.30 ± 0.06	0.42 ± 0.07	0.39 ± 0.03
Lung	0.74 ± 0.15	0.37 ± 0.04	0.45 ± 0.07	0.34 ± 0.02
Stomach	0.70 ± 0.15	0.43 ± 0.07	0.46 ± 0.05	0.20 ± 0.03
Duodenum	0.29 ± 0.07	0.31 ± 0.01	0.34 ± 0.04	0.28 ± 0.04
Ileum	0.48 ± 0.09	0.17 ± 0.03	0.21 ± 0.02	0.14 ± 0.02
Liver	0.38 ± 0.09	0.06 ± 0.03	0.20 ± 0.03	0.20 ± 0.01
Spleen	0.25 ± 0.04	0.22 ± 0.02	0.29 ± 0.04	0.23 ± 0.02
Bone marrow	0.16 ± 0.03	0.09 ± 0.03	0.15 ± 0.03	0.40 ± 0.01
Testes	—	0.39 ± 0.04	0.45 ± 0.07	0.37 ± 0.02
Skin	—	0.38 ± 0.06	0.42 ± 0.10	0.32 ± 0.03
Carcass	—	0.25 ± 0.04	0.21 ± 0.02	0.18 ± 0.003

[a] Values represent mean ± SE as percentage of the initial dose from four rats per treatment after 72 hr.
[b] Not determined.

Table 3. Metabolism of chlorine dioxide, chlorite and chlorate in rat urine.

Treatment	Percentage of initial dose[a]		
	Cl^-	ClO^-_2	ClO^-_3
$^{36}ClO_2$ (100 mg/l.)	26.93 ± 1.6	3.46 ± 1.0	0.73 ± 0.73
$^{36}ClO_2^-$(100 mg/l.)	31.55 ± 4.5	6.0 ± 0.6	—[b]
$^{36}ClO_3^-$(5 mg/l.)	20.50 ± 0.7	3.95 ± 2.7	8.2 ± 2.9

[a] Values represent the mean ± SE as percentage of the initial dose from four rats per treatment, throughout the 72 hr studied.
[b] None detected.

the initial dose. The distribution of $^{36}ClO_2$ was highest in the kidney, followed by lung, plasma, stomach, ileum, liver, duodenum, spleen and bone marrow, while the distribution of $HO^{36}Cl$ was highest in plasma, followed by bone marrow, kidney, testes, lung, skin, duodenum, spleen, stomach, liver, carcass and ileum. The distribution of $^{36}ClO_2^-$ was highest in plasma, followed by stomach, testes, skin, lung, duodenum, kidney, carcass, spleen, ileum, bone marrow and liver. In the case of $^{36}ClO_3^-$, plasma was highest, followed by stomach, lung, testes, kidney, skin, duodenum, spleen, ileum, carcass, liver and bone marrow.

In whole blood, when plasma was separated from packed cells, radioactivity was divided more or less evenly between the plasma and packed cells (0.55 ± 0.038 and 0.63 ± 0.11% of initial dose, respectively) for $^{36}ClO_2^-$, whereas for $^{36}ClO_3^-$, radioactivity preferentially accumulated in the plasma (0.68 ± 0.09% of initial dose vs. 0.23 ± 0.02% in the packed cells).

Table 4. Excretion of chlorine dioxide, chlorite, chlorate and chlorine in the rat.

Treatment	Percentage of initial dose[a]	
	Urine	Feces
$^{36}ClO_2$ (100 mg/l.)	30.81 ± 0.8	10.10 ± 1.7
$^{36}ClO_2^-$(10 mg/l.)	34.51 ± 1.4	4.75 ± 1.0
$^{36}ClO_3^-$(5 mg/l.)	40.14 ± 2.14	3.14 ± 1.0
$HO^{36}Cl$ (250 mg/l.)	21.52 ± 2.51	7.09 ± 0.24

[a] Values represent the mean ± SE as percentage of the initial dose from four rats per treatment, throughout the 72 hr studied. ^{36}Cl was not detected in expired air.

Metabolism of Cl Compounds

The administered ^{36}Cl compounds are found as various metabolites in rat urine, as shown in Table 3. After 72 hr from the time of administration, $^{36}ClO_2$ and $^{36}ClO_3^-$ are found in urine as Cl^-, ClO_2^- and ClO_3^- whereas $^{36}ClO_2^-$ is found as Cl^- and ClO_2^-. In all three cases, Cl^- is the major metabolite (approximately 87, 84 and 64% of the total radioactivity found in urine for $^{36}ClO_2$, $^{36}ClO_2^-$ and $^{36}ClO_3^-$, respectively).

Excretion of Cl Compounds

The total of the ^{36}Cl compounds recovered in the urine and feces 72 hr after administration was determined for each compound and calculated as the percentage of the initial dose. In the case of $^{36}ClO_2$, 75% of the recovered dose was found in the urine, while 25% was found in the feces. For $^{36}ClO_2^-$, 87 and 13% was found in urine and feces, respectively; and for $HO^{36}Cl$, 76 and 24% was found in urine and feces, respectively (Table 4).

Table 5. Effect of chlorine dioxide, chlorite and chlorate on the formation of chloroform in rat blood and organs.

Treatment[a]	Concentration, mg/l.	*n*	Organ	Effects
ClO_2	10	4	Blood	No effect
	100	7	Blood	Increased
	10	4	Liver, kidney, spleen, brain	No effect
	10	4	Testes	Increased
	100	7	Liver, testes, brain	Increased
ClO_2^-	100	7	Blood	No effect
	100	7	Liver, brain	Increased
ClO_3^-	100	9	Blood	No effect
	100	9	Liver	Increased

[a] Rats were treated with chlorine dioxide and its metabolites daily for one year.

^{36}Cl compounds were not found in the expired air of any treatment group throughout the 72 hr time period.

Effect of Cl Compounds on the Formation of Chloroform in Rat Blood and Organs

Table 5 shows the effects of chronic treatment with chlorine dioxide and its metabolites for one year on the formation of chloroform in rat blood, liver, kidney, spleen, brain and testes. A concentration of 100 mg/l. ClO_2 daily in the drinking water caused an increase in chloroform levels in blood, liver, testes and brain. A concentration of 10 mg/l. ClO_2 increased chloroform levels in rat testes, while levels in blood, liver, kidney, spleen and brain remained unchanged. Treatment of rats with 100 mg/l. ClO_2^- for one year elevated chloroform levels in the liver and brain, while the levels in blood were not affected. Treatment with 100 mg/l. ClO_3^- increased liver but not blood chloroform concentrations after one year.

Discussion

From the kinetics study, it is apparent that ClO_2 and its metabolites (ClO_2^- and ClO_3^-) are more rapidly absorbed into the bloodstream than HOCl. The effect is very pronounced when comparing ClO_2 itself with HOCl. Likewise, HOCl is more slowly eliminated from rat plasma than ClO_2 or its metabolites.

The distribution study revealed that although plasma and whole blood contain the highest activity, the bone marrow contained far less in $^{36}ClO_2$, $^{36}ClO_2^-$ and $^{36}ClO_3^-$ treatment, while HOCl had a higher activity in bone marrow. The high activity in testes, even after 72 hr, indicates a possible pharmacologic action at this site. Effects of these compounds on the reproductive system are currently in progress in our laboratory.

It is important to indicate that after 72 hr, high amounts of radioactivity were still found in the stomach, duodenum and ileum.

The Cl compounds studied are eliminated mainly by kidney and intestinal routes, since no radioactivity was found in expired air. For $HO^{36}Cl$ and $^{36}ClO_2$ treatment, about 75% of the recovered dose was found in the urine; in the case of $^{36}ClO_2^-$ and $^{36}ClO_3^-$, about 90% of the recovered dose was in the urine.

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Environmental Health Perspectives
Vol. 46, pp. 25-29, 1982

Assessment of Maternal Toxicity, Embryotoxicity and Teratogenic Potential of Sodium Chlorite in Sprague-Dawley Rats

by Daniel Couri,* Carl H. Miller, Jr.,* Richard J. Bull,* John M. Delphia† and Elsayed M. Ammar*

Groups of up to 13 pregnant rats were individually caged. Body weight, food and water consumption were recorded at days 1, 8, 15 and 22 of gestation and the dams were treated on days 8–15 with sodium chlorite, 0.1%, 0.5% or 2% in drinking water or by injection of 10, 20, or 50 mg/kg IP or by gavaging with 200 mg/kg. To prevent ingestion of stillborn pups some dams were sacrificed at day 22. Other dams were allowed to deliver at term. Fetuses were weighed, measured and examined for soft tissue and skeletal malformations. Sodium chlorite, 20 or 50 mg/kg daily IP or gavaging with 200 mg/kg, caused vaginal and urethral bleeding. Doses of 10, 20 or 50 mg/kg daily IP caused 0, 50 and 100% mortality of dams, respectively. No deaths were caused by soduim chlorite in the drinking water, but the dams' body weight, water and food consumption decreased during all treatments except 0.1% in the drinking water. Blood smears from the dams injected IP or drinking 2% sodium chlorite showed irregular, bizarre and ruptured erythrocytes. Injection of 10 or 20 mg/kg or drinking 2% resulted in decreased litter size and increased stillbirths and resorption sites. Drinking 0.1% or 0.5% sodium chlorite did not produce any significant embryotoxicity. With all treatments, no significant gross soft tissue or skeletal malformations were observed. Postnatal growth of the pups was not affected by any treatment of the dams during the gestation period.

Introduction

The chlorite ion is formed in water from chlorine dioxide treatment. Since chlorine dioxide is under investigation as an alternative to chlorine disinfectant in the U.S., the toxicological profile of chlorine dioxide and its products in water must be thoroughly investigated. Some hematologic aspects of chlorine dioxide and sodium chlorite toxicity have been assessed *(1–3)*. However, the health effects of chlorine dioxide or its products chlorite and chlorate have not yet been investigated during pregnancy. In this study we aimed to investigate the maternal toxicity, embryotoxicity and teratogenic potential of sodium chlorite in a pregnant rat model. Sodium chlorite was administered at various dose levels and by various routes of administration during the period of organogenesis in pregnant Sprague-Dawley rats. The parameters examined for assessment of chlorite toxicity in pregnant rats were maternal lethality or other toxic manifestation, changes in body weight, food and water consumption, fetal viability, size, weight, soft tissue and skeletal malformation.

Materials and Methods

Virgin female Sprague-Dawley rats weighing 268–303 g and fertile male Sprague-Dawley rats weighing 300–320 g were obtained from the Laboratory Supply Company, Indianapolis, Indiana.

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The rats were housed in the Ohio State University College of Medicine Vivarium and were given tap water and Purina Lab Chow ad libitum for one week for acclimation before the experiments began. During the course of the study rats were maintained at 55% RH, 73–74°F and a 12-hr light-dark cycle begining at 6:00 a.m.

Four female rats were mated with one male rat in the late afternoon and on the next morning female rats were checked for positive mating. The day of finding a vaginal plug and/or sperm in the vaginal smear examined under light microscopy was considered day 1 of gestation.

Rats verified for conception were weighed on day one and housed individually in cages provided with double distilled water and food ad libitum. The weights of pregnant rats as well as their water and food consumption were monitored at days 8, 15, and 22 of gestation. One group of rats was maintained on doubly distilled water till delivery to serve as the control. Other groups were given several treatments with sodium chlorite either by intraperitoneal injection, gavage or in drinking water during gestation period days 8–15. Sodium chlorite flakes ($NaClO_2$, MW 90.45) were obtained from Matheson, Coleman and Bell Manufacturing Chemists, Norwood, Ohio.

Sodium chlorite was administered to groups of rats IP at dose levels of 50, 20 and 10 mg/kg daily; by gavage at a dose of 200 mg/kg daily and in drinking water ad libitum at concentrations of 2%, 0.5% and 0.1%. Sodium chlorite solutions for drinking were replaced three times during the period of treatment (days 1, 3 and 5) with freshly prepared solutions. General health and toxic manifestation to the dams were observed during treatment and until delivery.

Some pregnant rats surviving treatment were allowed to deliver at term. Litter size, number of living and dead pups, weights of pups and crown-rump measurements were recorded. Offspring were examined for gross malformations and then killed with ether and divided into two groups. One group of pups was preserved in Bouin's solution for examination of soft tissue malformation under a dissecting microscope by the serial sectioning technique *(4,5)*. The other group of pups was fixed in 95% ethyl alcohol for skeletal examination after clearing of the specimens and staining with alizarin red *(6,7)*.

Since dams tend to cannibalize malformed or dead pups, other groups of pregnant rats were subjected to cesarean section before parturition at day 22. Dams were anesthetized with ether and the abdominal cavity was opened to expose the uterine horns which were longitudinally incised. Live, dead and resorbed fetuses were counted. Living fetuses were distinguished by the appearance of reflex motion after touching the fetus with forceps. Fetuses were then separated from the placenta, dried with filter paper, weighed and the crown–rump distance measured. As mentioned above, fetuses were prepared for gross, soft tissue and skeletal examination for malformation. The uterine horns were stained to reveal sites of early resorption *(8)*.

Blood samples were withdrawn from sacrificed dams by heart puncture and blood smears were stained with Wright stain to examine blood cells under light microscopy.

Postnatal growth was monitored by selecting six pups at random from each litter and weighing them at days 1, 8, 15, 22 and 29.

Statistical Analysis

A Hewlett-Packard 9825A and software Volume I was used to calculate significance according to Student's *t*-test or chi square. When the F test indicated the *t*-test could not be used with confidence, the normal curve and the larger standard deviation were used to estimate significance.

Results

Maternal Toxicity

The toxic effects and lethality of sodium chlorite administered to pregnant rats during gestation are summarized in Table 1. Sodium chlorite administered IP at a dose of 50 mg/kg produced profuse vaginal and urethral bleeding and 100% mortality within 2 days. The dams decreased in weight by 7%. However, the gain in weight during days 1–8, a no-treatment period, was 98%. The uterine horns were found to be implanted with an average of 12.4 embyros per litter. At a dose of 20 mg/kg IP, the mortality rate was 50%; vaginal and urethral bleeding as well as weight loss (19.5%) were also observed. At a dose of 10 mg/kg IP, sodium chlorite did not cause lethality or bleeding, but loss of weight was still observed. Effects of gavaging with 200 mg/kg sodium chlorite were similar to effects of 50 mg/kg IP. The various concentrations of sodium chlorite 2%, 0.5% and 0.1% in drinking water did not cause any deaths.

Effects of Sodium Chlorite on Dams' Body Weight, Food and Water Consumption

The changes in dams' body weights, food and water consumption are shown in Table 2. It is evident that the dams' body weight decreased during all treatments with sodium chlorite except 0.1%.

Table 1. Toxicity of sodium chlorite in pregnant Sprague-Dawley rats.

NaClO$_2$ treatment	Number of rats in group	Route of administration	Treatment period, gestation days	Toxic effects observed	Lethality, %
[illegible] mg/kg	7	IP	8-10 or 16-17	Vaginal, urethral bleedings; loss of weight	100
[illegible] mg/kg	10	IP	8-15	Vaginal, urethral bleedings; loss of weight	50
[illegible] mg/kg	13	IP	8-15	Loss of weight	0
20[illegible] mg/kg	4	PO Gavaging	8-10	Vaginal, urethral bleedings; loss of weight	100
2%	10	PO Drinking water	8-15	Loss of weight	0
[illegible]5%	10	PO Drinking water	8-15	Loss of weight	0

Table 2. Changes in dams' body weight, food and water consumption during and after treatment with sodium chlorite.

	Treatment on Gestation Days 8-15[a]			Recovery on Gestation Days 15-22[a]		
Treatment	Body weight change, g/rat	Food consumed, g/rat	Water consumed, ml/rat	Body weight change, g/rat	Food consumed, g/rat	Water consumed, ml/rat
Double distilled water (control)	27.20 ± 7.63	166.20 ± 10.66	353.60 ± 27.98	32.80 ± 1.24	113.20 ± 14.90	284.80 ± 19.29
2% NaClO$_2$ in drinking water	–72.50 ± 6.18[b]	54.50 ± 5.80[b]	63.22 ± 6.52[c]	98.00 ± 9.17[c]	118.50 ± 8.190[e]	276.11 ± 8.23[e]
0.5% NaClO$_2$ in drinking water	–18.75 ± 21.40[d]	112.75 ± 18.32[c]	224.37 ± 51.78[d]	86.71 ± 7.96[c]	139.50 ± 6.60[d]	264.87 ± 14.68[e]
0.1% NaClO$_2$ in drinking water	71.11 ± 16.60[d]	168.44 ± 3.19	237.55 ± 8.33[b]	14.44 ± 17.15[d]	147.11 ± 7.52[d]	292.22 ± 13.03[e]
10 mg/kg NaClO$_2$ IP	3.69 ± 6.25[b]	129.43 ± 10.07[d]	249.54 ± 10.97[b]	74.77 ± 9.92[d]	141.50 ± 6.27[d]	286.31 ± 9.21[e]
20 mg/kg NaClO$_2$ IP	–53.20 ± 7.58[b]	59.0 ± 10.89[b]	203.00 ± 26.25[c]	74.00 ± 28.59[d]	157.60 ± 11.15[d]	312.00 ± 25.72[e]

[a] Data represent means ± SE of 7-13 animals. On day 1, mean weight of rats in various groups ranged between 268 and 293 and the differences were not significant.

[b] $p < 0.001$.

[c] $p < 0.01$.

[d] $p < 0.05$.

[e] $p > 0.05$.

The increase in body weight under the influence of 0.1% sodium chlorite was significantly higher than the control group maintained on double distilled water. All sodium chlorite treatments whether in drinking water or by IP injection significantly decreased water consumption during the treatment period. Food consumption was decreased during all treatments except 0.1% sodium chlorite in drinking water.

During the period following the end of treatment until day 22 (recovery, Table 2) there was no significant difference in water consumption between treated and control rats. Food consumption was also increased for all treated groups, except the groups which drank 2% sodium chlorite or 0.1% sodium chlorite. During the recovery period the body weight gain for those groups which lost weight during treatment was significantly higher than the body weight gain in the control group.

Effect of Sodium Chlorite Treatment on Red Blood Cells of Dams

Blood smears from dams sacrificed at day 22 showed irregular blood cells, ruptured cells and hemolysis. This was observed only in rats which received 20 mg/kg IP and in those which drank 2% sodium chlorite.

Effect of Sodium Chlorite Treatment on Embryo and Fetus

The litter size, number of live and stillborn fetuses, the fetal weight and crown-rump measurements of litters delivered at term are shown in Table 3. With 2% sodium chlorite in drinking water, as well as with 10 mg/kg IP treatment of the dams, the litter size decreased and the number of stillbirths increased, but the changes were not statistically significant ($p > 0.05$) by the *t* test. The crown-rump distance was significantly decreased under all treatments. However the fetal weight was not significantly decreased. Table 4 shows the effect of sodium chlorite treatment on fetuses of rats sacrificed at day 22. It is evident that the higher concentrations of sodium chlorite increased the number of dead and resorbed fetuses and decreased both the number of live fetuses and the crown-rump distance. There were three whole litter resorptions out of five treated

Table 3. Effect of administration of sodium chlorite to pregnant Sprague-Dawley rats during day 8-15 of gestation on tters delivered at term.

Treatment	Litter size^a		Crown-rump, length, cm^a	Fetus weight,
	Alive	Stillborn		
Double distilled water (control)	9.80 ± 1.11	0	4.44 ± 0.07	7.24 ± 0.[illegible]1
2.0% $NaClO_2$ in drinking water	6.5 ± 2.50	2.0	4.18 ± 0.05^b	6.52 ± 0.[illegible]2
0.5% $NaClO_2$ in drinking water	11.75 ± 0.75	0	4.30 ± 0.06^b	6.54 ± 0.[illegible]2
0.1% $NaClO_2$ in drinking water	9.80 ± 0.40	0.5	4.22 ± 0.02^b	6.85 ± 0.[illegible]
10 mg/kg IP	8.86 ± 0.99	1.14	4.27 ± 0.05^b	6.61 ± 0.[illegible]2

[a] Data represent means ± standard error of 4 to 7 litters.
[b] $p < 0.05$.

Table 4. Effect of administration of sodium chlorite to pregnant Sprague-Dawley rats during day 8-15 on fetuses delivered t day 22 by cesarean section.

Treatment	No. of fetuses			Crown-rump length cm^a	Fetus weight, g^a
	Alive	Dead	Resorbed		
0.1% $NaClO_2$ in drinking water (control)	40^b	1^b	4^b	3.66 ± 0.03	4.14 ± 0.65
0.5% $NaClO_2$ in drinking water	44^b	1^b	2^b	3.87 ± 0.05^d	5.23 ± 0.14
2.0% $NaClO_2$ in drinking water	37^b	2^b	21^b	3.37 ± 0.10	4.59 ± 0.19
10 mg/kg $NaClO_2$ IP	58^c	3^c	7^c	4.06 ± 0.04^d	5.35 ± 0.14
20 mg/kg $NaClO_2$ IP	15^c	0^c	34^c	—	—

[a] Data represent means of 4 or 5 litters.
[b] $p < 0.005$ by X^2.
[c] $p < 0.0001$ by X^2.
[d] $p < 0.05$ by t test.

Table 5. Postnatal growth of pups delivered to dams treated with sodium chlorite during days 8 to 15 of gestation.

Treatment	Postnatal day^a					Postnatal mortality
	1	8	15	22	29	
Double distilled water (control)	42.8	113.8	209.4	274.3	456.7	0
2% $NaClO_2$ in drinking water	39.8	108.4	200.1	274.4	492.2	0
0.5% $NaClO_2$ in drinking water	38.2	102.4	209.0	326.7	484.5	0
10 mg/kg $NaClO_2$ IP	40.5	110.7	215.7	332.9	496.3	0

[a] Data represent mean weight in grams of 6 pups selected at random from each of 4 or 5 litters.

with 20 mg/kg IP, two litters out of five from dams drinking 2% sodium chlorite and one whole litter resorbed out of five dams given 10 mg/kg IP.

Effect of Maternal Treatment with Sodium Chlorite on the Postnatal Growth of Pups

The postnatal weights of six pups from each litter is shown in Table 5. There was no significant difference in the weight of pups delivered by control and treated dams.

Effect of Sodium Chlorite Treatmen on Soft Tissues and Skeletal Format on of the Fetus

The incidence of soft tissue and skeletal ma lformations in the control group and sodium chl rite treatment was not significantly different and falls within the normal range of spontaneous malfo mation in rats.

Discussion

Sodium chlorite administered to pregnant Spra gue-Dawley rats exerted toxic effects when given e ther

Table 6. Calculated daily dose of sodium chlorite administered to pregnant rats in drinking water.

$NaClO_2$ treatment	Sodium chlorite, mg/rat/day
Control	0
2%	212
0.5%	163
0.1%	34

by intraperitoneal injection, by gavage or in drinking water. The injection of 50 mg/kg or 20 mg/kg daily resulted in a cumulative toxic effect which caused 100% and 50% mortality, respectively. Gavaging of 200 mg/kg daily also produced 100% mortality. Injection of sodium chlorite as well as administration of sodium chlorite in drinking water in concentrations higher than 0.1% caused body weight loss and decreased food and water consumption during the treatment period, days 8–15 of gestation. However, after stopping sodium chlorite treatment, the body weight gain was more than the controls, probably as a compensatory mechanism. Decreased food intake continued even after cessation of treatment which may indicate a delayed toxic effect on the dams. Blood smears of rats receiving the higher concentrations of sodium chlorite, 20 mg/kg IP and 2% in drinking water, showed hemolysis of red blood cells.

The embryos and fetuses were affected by sodium chlorite treatment in higher concentrations administered either IP or in drinking water. The toxic effects on the fetus include decreased litter size, increased stillbirths and increased resorption of embryos. Whole litter resorption was observed in dams treated with the 2% sodium chlorite in drinking water and in those injected with 10 and 20 mg/kg IP. This, coupled with the observation of vaginal bleeding during treatment, may indicate an early abortifacient effect of higher doses of sodium chlorite. The toxic effect on the fetus may be due to hypoxia produced as a result of methemoglobinemia (*1-3*) and hemolysis. However, a direct toxic effect on the embryo cannot be excluded since results from this laboratory have shown that sodium chlorite is embryotoxic in the early stage of chick embryo development (*9*).

Teratogenic effects of sodium chlorite were not reported. We feel that expanded studies with sodium chlorite at other concentrations are needed to shed light on the embryotoxicity of sodium chlorite. The route of administration is crucial in assessment of the embryotoxicity of sodium chlorite as Table 6 indicates that the calculated amounts of sodium chlorite administered to rats daily were higher by the oral route than by injection. However, the injected sodium chlorite produced a higher maternal and embryotoxicity. Also, gavaging 200 mg/kg produced more toxicity than higher amounts given ad libitum in drinking water.

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Environmental Health Perspectives
Vol. 46, pp. 31-37, 1982

Toxicological Effects of Chlorite in the Mouse

by Gary S. Moore* and Edward J. Calabrese*

When exposed to a maximum level of 100 ppm chlorine dioxide in their drinking water, neither A/J or C57L/J mice exhibited any hematologic changes. Chlorite exposure under similar conditions produced increases for red blood cells in osmotic fragility, mean corpuscular volume, and glucose-6-phosphate dehydrogenase activity for both strains. Chlorite exposure of pregnant A/J mice resulted in a significant decrease in the weight of pups at weaning and a lower average birth to weaning growth rate. Mice exposed to as much as 100 ppm sodium chlorite ($NaClO_2$) in their drinking water for up to 120 days failed to demonstrate any histopathological changes in kidney structure.

Introduction

The primary method of water disinfection in the United States employs chlorine. Studies, however, have shown that chlorine interacts with organics in the water to form trihalomethanes (*1*,*2*). One of the trihalomethanes is chloroform, which has been found to be carcinogenic in rats (*3*). It has been postulated that chlorinated water supplies may increase the risk of cancer to humans (*4*–7); such compounds are formed to a lesser extent in water disinfected with chlorine dioxide (*8*). While toxicological studies have revealed limited physiologic effects from chlorine dioxide, concern has now been directed towards the end products of such disinfection which include chlorate and chlorite (*7*,*8*). Chlorites appear in concentrations of up to 50% of the chlorine dioxide demand where chlorine dioxide is used for disinfection (*8*). Blood destruction, nephritis, and methemoglobinemia have developed in humans after acute poisoning with large doses of chlorate (*9*,*10*). It was suggested that in chronic poisoning from chlorate, uremia, anemia, and nephritis might develop. Chlorite is thought to be a more potent oxidant stressor to blood than chlorate (*11*). Heffernan (*12*,*13*) reported that chlorite belonged to a class of oxidant compounds that stimulate the reduced glutathione (GSH) levels and the development of hemolytic anemia in animal models.

In attempting to establish minimal health effect levels of oxidants (chlorine dioxide and chlorite) on erythrocytes, it is important to consider the effects of such stressors on potential high risk groups. A large group that falls into this category are persons with lowered G6PD (glucose-6-phosphate dehydrogenase) activity. G6PD-deficient cells have a reduced ability to produce NADPH via the pentose phosphate pathway (PPP), and consequently less GSH is formed. Since GSH is the primary mechanism of defense of the red blood cell against oxidant stress, then persons with deficient G6PD levels have a lowered capacity for protection against oxidants (*14*, *15*) and virtually 100 million males throughout the world belong in this category (*14*). Among the studies reported here are those designed to evaluate the effects of chlorine dioxide and chlorite on erythrocytes of mouse strains with differential G6PD activity (i.e., the A/J strain has three times the activity of the C57L/J strain).

Another potential high risk group would likely include newborns. The potential effects of chlorite on human newborns may be exaggerated because: (1) infants consume nearly three times as much liquid per unit of body weight than adults (*16*); (2) infants are born with hemoglobin F (fetal hemoglobin), a form that is readily oxidizable to methemoglobin (*17*); (3) infants have a lower capacity than adults to reduce methemoglobin enzymatically to

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hemoglobin (*18*); (4) newborn infants are characteristically low in vitamin E (*18*), an important antioxidant compound, because there is little placental transfer of vitamin E.

There is also evidence that the fetus may be at increased risk to oxidant stressors. Shuval and Gruener (*19*) demonstrated that nitrites can be transferred to the fetus *in utero* causing an elevation of MetHb in fetal blood. When administered in drinking water to pregnant rats, 2000–3000 mg/l. of $NaNO_2$ resulted in a decreased litter size, increased mortality of pups in the first three weeks and a lagging of growth rate despite having equal birthweight to controls.

Sodium chlorate has been shown to produce methemoglobin *in vitro* and *in vivo* when administered orally (*9,10,20*). Oral acute doses of sodium chlorate have resulted in hemolysis, nephritis, methemoglobinemia, and death in man and various species of test animals (*9*). Since previous studies have shown chlorate to cause renal damage, it was hypothesized that chlorite, because of its more potent oxidant capabilities, may also be potentially harmful to the kidney.

In order to test the above hypotheses, a number of experiments were performed on laboratory animals. The experiments were designed to measure the effects of orally administered chlorite on blood characteristics, kidney structure, as well as the fertility, and the growth rate, mortality and development of newborns.

Methods

Animal Care

Two strains of mice, with "normal" (A/J) and low (C57L/J) levels of G6PD activity, were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were individually housed in stainless steel cages equipped with constant feed/food hoppers and 50 ml calibrated watering devices. The cages were kept under controlled conditions of 12 cycles of light and dark (9 A.M. - 9 P.M. light, 9 P.M. - 9 A.M. dark), and a temperature of 74 ± 2°F. Animals were held at these conditions for a one-week period after receipt in order to assess their health and allow for acclimation. Animals were randomly allocated to each of the treatment categories, according to a random scheduling of treatments. Treatment periods of 12 hr occurred during the dark cycle in order to minimize decomposition of ClO_2 by light and to coincide with the nocturnal activity period of the mice.

The watering devices were filled with the appropriate treatment solutions and a reading taken of the volume both before and after the treatment period. The bottles were removed at the end of this period, and no other water provided until the next treatment period. Average daily consumption of liquid was computed for each mouse, and an overall average computed for each treatment category.

Mice were fed *ad libitum* a balanced rodent diet (Purina Rodent Chow) composed of not less than 23% crude protein, 4.5% crude fat, 6.0% crude fiber, 8.0% ash, and 2.5% minerals.

Experimental Procedures

Complete Blood Count (CBC). The Royco Cell-crit IV Hematology System, consisting of Model 920-A cell counter, Model 720-A hemoglobinometer and Model 365-A diluter, was used to determine red blood cell count (RBC), hematocrit (HCT), white blood count (WBC) and hemoglobin concentration (HBG). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were then computed from these data.

Quality assurance of equipment performance was checked on a daily basis by means of commercial standards, and equipment performance was measured by comparison of automated and manual hematocrits of the same blood sample.

Reticulocyte Count. Staining of reticulocytes was accomplished according to the procedure outlined by Seiverd (*21*). A 1% physiological saline solution of Brilliant Cresyl Blue was mixed with an equal quantity of whole blood and allowed to stand for 20–30 min. The contents of the tube were then remixed and a smear made. The smear was observed under oil immersion and a total of 500 cells counted. The reticulocytes were expressed as a percentage of the total number of cells counted.

Glucose-6-Phosphate Dehydrogenase Assay (G6PD). G6PD activity was determined by a colorimetric procedure using Calibiochem (10933 N. Torrey Pines Rd. LaJolla, Calif. 92037) G6PD reagents and a Perkin-Elmer Coleman 55 spectrophotometer equipped with a flow-through temperature-controlled cell, a Coleman 5–100 enzyme calculator, and a Coleman 47 B.C.D. printer. The G6PD activity was calculated in terms of International Units (I.U.) per gram of hemoglobin in whole blood hemolysate.

Osmotic Fragility (Automated). Osmotic fragility was determined using the American Optical Fragilograph (Clinical Instruments Co., Dudley, MA 01570). A 10 µl portion of whole blood was diluted in 7.5 ml of 0.68% isotonic buffered saline (Isoton II diluted to obtain 0.68%), yielding a 1:750 dilution. The instrument was calibrated with 1.5 ml of the prepared cell suspension, added to a cuvette, and the osmotic fragility determined.

***Glutathione (GSH) Assay*.** Reduced glutathione was determined by using Glutathione and Erythrozyme Assay (from Princeton Biomedix Inc., Princeton, NJ) using a reagent solution containing 5,5-dithiobis(2-nitrobenzoic acid) (DTNB) which reacts with glutathione to form a highly colored yellow ion.

Statistical Analysis

Analysis was performed by using the BMDP2V and SPSS ONEWAY (*22*) programs. The two-way ANOVA table was produced by the BMDP program with the three previously mentioned hypotheses either accepted or rejected, based upon the p values of the F test ($p = 0.05$). If a hypothesis was rejected, contrasts were constructed and with separated variances computed with accompanying p values. These contrasts were run on the SPSS ONEWAY program (*22*). The Bartletts and Cochrans tests for equal variances were also computed by using the SPSS ONEWAY program.

Experimental Design

***Effect of Chlorite on Chlorine Dioxide on Blood Characteristics*.** Twenty A/J and 20 C57L/J mice were each divided into two groups of 10 mice with each strain being exposed to 0.0 ppm (10 mice) and 100 ppm (10 mice) chlorine dioxide in their drinking water for 30 days. The animals were lightly etherized and then decapitated by means of a guillotine. The blood was collected into 100 µl of 0.6% sodium heparin (Sigma) prior to analysis.

Both A/J ($N = 63$) and C57L/J ($N = 59$) mice were each divided into four groups and exposed to sodium chlorite at 0.0, 1.0, 10.0 and 100.0 ppm in their drinking water for 30 days (Table 1). At the end of the exposure period, blood was collected and analyzed as previously indicated. In addition to measuring the effects of several blood parameters including GSH levels, the weight change over 30 days and the average volume of water consumed per day was determined for each animal.

***Effect on Conception Rate and Litters of A/J Strain Mice*.** Ten- to 12–week-old female A/J mice were grouped by ten in an 11.5 in. long × 7.5 in. wide × 5 in. deep polycarbonate breeding cage for one week in order to synchronize ovulation. Females were then randomly mated one to one with A/J males of a similar age and checked for vaginal plugs on the mornings of the next four days. Males were removed from females at the appearance of plugs. Date of plugging, as well as weight of sire and dam were recorded. Females that were not plugged were grouped for a week before breeding again.

Plugging was considered to be day one of pregnancy and females were placed in fresh breeding cages with wood chips for the duration of their pregnancy and lactation. At plugging, females were randomly assigned to either a control group which was given distilled water or a treatment group which was given 100 ppm sodium chlorite (Matheson, Coleman and Bell, Norwood, OH 45212) in distilled water. A dose of 100 ppm ClO_2^- was selected, since other studies (*12,13,23*) have shown that effects on hematological parameters are first observed at concentrations of chlorite reaching 50 to 100 ppm. Females remained on the specified water during gestation and lactation periods, and water intake was monitored during this time.

At parturition each litter was examined and data collected. Individual birth weights were taken and pups were weighed once a week until weaning at 28 days. Statistical analysis was performed on the data collected for each litter as well as water consumption and rate of positive pregnancy for each group.

***Effects of Renal Structure*.** Water used for all treatment and control groups was processed by a Continental Water Deionization System (Millipore Corp., Bedford, Mass) and buffered with 1/30 *M* Sorenson's phosphate buffer (*24*) to maintain a pH of 7.17. Treatment water was prepared by the addition of appropriate amounts of $NaClO_2$ (Matheson Coleman and Bell, Norwood, Ohio) to buffered "Continental" water. Water used for control groups was prepared in a similar fashion with the addition of NaCl (Sigma Chemical Co., St. Louis, MO) instead of $NaClO_2$. All water for treatment and control mice was available *ad libitum*, and the amount of water consumed was recorded at every fresh change. Measurement of chlorite ion concentration in freshly prepared treatment water was performed using the DPD ferrous titrimetric method of Palin (*25*).

All mice were weighed and randomly allocated to specific time-exposure and treatment groups. Lengths of exposures of treatment and control mice were divided into three time-exposure periods: one group of 55 mice for 30 exposure days, one group of 55 mice for 90 exposure days, and one group of 60 mice for 180 exposure days. The five treatment groups within each time-exposure group were: 0.0 ppm $NaClO_2$ as a negative control, 4.0 ppm $NaClO_2$ (2.98 ppm as ClO_2^-), 20.0 ppm $NaClO_2$ (14.92 ppm as ClO_2^-), 100.0 ppm $NaClO_2$ (74.59 ppm as ClO_2^-), and 100.0 ppm sodium chloride (NaCl) as a high salt control.

Upon completion of their respective treatment periods, all animals were weighed and killed by cervical dislocation. Both kidneys were excised, weighed, divided longitudinally into two hemisections, and placed in appropriate fixatives for subsequent light microscopy and/or transmission electron microscopy. In preparation for light microscopy, portions of renal tissue were fixed for 12 hr in Helly's solution and subsequently chromated for 12 hr in a 3% potassium dichromate solution (*24*). Kidney hemisections were embedded in paraffin, cut in 5 μm sections, and stained with hematoxylin and eosin-phloxine B. Renal tissue from three randomized mice in each group were processed for electron microscopy. Portions of renal tissue were cut in 1.5 cm blocks and fixed overnight at 4°C in 2.6% glutaraldehyde in Millonig's phosphate buffer at pH 7.2 (*26*). The renal tissue was then finely diced and post-fixed for 1 hr in Millonig-buffered 1% osmium tetroxide solution. After Epon embedding and ultrathin sectioning, the tissues were stained with 7% uranyl acetate and lead citrate (*27*). Electron microscopy was performed by use of a Joel 100–S electron microscope at 80 kV.

Results

Effect of Chlorine Dioxide and Chlorite on Blood Characteristics

A total of 11 hematologic parameters were measured for effects of chlorine dioxide exposure. Mice exposed to sodium chlorite for 30 days exhibited treatment effects for three of the eleven hematologic parameters (Table 1). Specifically, there was an increase in G6PD activity, MCV (mean corpuscular volume) and osmotic fragility for both strains of mice exposed to the 100.0 ppm chlorite, whereas no significant differences from controls occurred for the 1.0 and 10.0 ppm chlorite exposures.

It is also evident from analysis of variance (Table 1) that there is a significant difference between strains for both G6PD activity ($p < 0.001$) and osmotic fragility ($p < 0.001$). There were no significant strain versus treatment interactions, indicating that both strains acted similarly to chlorite exposure with respect to the parameters measured. These differences in hematological parameters from treatment could not be explained by weight loss or gain ($p =$ 0.146), or by differences in water consumption ($p =$ 0.291) since there were no significant treatment effects with respect to these parameters.

Effect of Chlorite on Conception Rate and Litters of A/J Strain Mice

The means, standard deviations and *t*–results are presented for control and treatment groups in Table 2. The average number of pups born alive in each litter was 5.4 for controls and 5.0 for the treatment group ($p = 0.33$). The average number of pups alive at weaning (4 weeks after birth) was greater at 4.2 pups for controls than for the 3.4 pups in the treatment group ($p = 0.24$). In contrast to the number of living pups, the average number of pups per litter dead at birth was 1.5 for controls and 2.0 for treatment animals ($p = 0.25$). The average number of pups per litter that died after birth but before weaning was 1.3 for controls and 1.9 for the treatment group ($p = 0.214$).

The control litters had an average live birth litter weight of 1.27 g compared with 1.17 g for the treatment group ($p = 0.13$). Two variables where statistical significance was reached includes the averaged weight of pups at weaning and the average birth to weaning growth rate. The birth to weaning growth rate was 0.336 g/day for treatment animals while being significantly greater at 0.408 g/day for

Table 1. Analysis of variance and cell means for selected hematologic variables of A/J and C57L/J mice exposed to 0.0, 10.0, and 100.0 ppm of sodium chlorite.

		Cell means for			Analysis of variance results[a]			
Parameter	Treatment, ppm NaCl	A/J strain	Na	C57L/J strain	*N*	S	T	ST
G6PD activity, IU/g of hemoglobin in whole blood	0.0	6.10	16	1.88	14	0.000	0.000	0.90
	1.0	6.05	12	1.74	11			
	10.0	5.90	12	1.82	11			
	100.0	6.50	23	2.13	23			
Mean corpuscular volume	0.0	48.91	16	49.89	14	0.100	0.008	0.84
	1.0	48.70	12	49.47	11			
	10.0	48.58	12	49.25	11			
	100.0	50.39	23	50.55	23			
Osmotic fragility	0.0	0.440	12	0.458	11	0.000	0.084	0.87
	1.0	0.440	12	0.453	11			
	10.0	0.438	12	0.458	11			
	100.0	0.452	12	0.465	11			

[a] *N* = number of mice tested per cell. Probability *F* exceeded for strain differences (S); treatment (T) differences; and strain versus treatment (ST) interactions. When retested by orthogonal contrasting, significance was achieved ($p = 0.05$) for individual treatments.

Table 2. **Differences between control (0.00 ppm chlorite) and treatment (100 ppm chlorite) groups for ten variables.**[a]

Variable	Control group			Treatment group			t-test[b]		
	Mean	S	N	Mean	S	N	t	df	p
Liver litter size	5.4	2.5	21	5.0	3.3	12	0.42	31	0.339
No. alive at weaning	4.2	2.4	19	3.4	3.7	12	0.73	29	0.237
Avg. pup weaning weight	12.5	1.6	16	10.7	2.4	7	2.06	21	0.026
Birth-weaning growth rate	0.408	0.055	16	0.336	0.082	7	2.11	21	0.024
Gestation time	20.2	0.68	9	19.71	1.30	12	1.16[b]	17.1[b]	0.110
Breeding weight of mother	21.84	2.16	13	21.03	1.57	12	1.07	23	0.149
Age of mother at parturition (weeks)	18.0	3.9	21	18.5	4.21	12	–	–	–
Average liver birth litter weight	1.27	0.380	20	1.17	0.113	10	1.15[b]	24.7[b]	0.130
No. dead at birth	1.50	2.01	20	2.00	2.04	12	–0.68	30	0.252
No. survivors dying before weaning	1.32	1.77	19	1.90	2.02	10	–0.81	27	0.214

[a] S = standard deviation; N = number of litters.

[b] t-separate statistic, with adjusted degrees of freedom, are presented, since the variances of the control and treatment groups are shown to be unequal by the F-test (p = 0.10). Results are based on a one-sided test (H_A: mean for controls litters > mean for treatment litters).

Table 3. **Analysis of variance for body weight (BW) change, kidney weight (KW), kidney: body weight ratio and weekly water consumption.**

Parameter measured	No. of mice	Analysis of variance[a]		
		L	T	$L \times T$
Body weight change	167	0.000	0.690	0.808
Kidney weight	167	0.000	0.602	0.331
KW:BW ratio	167	0.103	0.348	0.602
H_2O consumption	166	0.023	0.948	0.519

[a] Probability F exceeded for length of exposure differences (L), treatment differences (T), and length of exposure versus treatment interactions ($L \times T$).

the control group (p = 0.024). Further, the average weight at weaning was 10.7 g for the treatment group and 12.5 g for the control group (p = 0.026).

The conception rate was also determined for control and treatment groups. All females positive for vaginal plugs were randomly placed into control and treatment groups. Not all plugged females conceived or produced litters. The percentage of dams that were plugged that also produced litters was defined as the conception rate. The conception rate for the treatment group was 39% and for controls was 56%.

Since differences between groups in the weight of dams, age, gestation time, or the amount of water consumed might have influenced the development of pups, these variables were measured. The gestation time of dams was not statistically different between control and treatment groups (p = 0.110). The average breeding weight of the treatment and control dams was 21.03 and 21.84 g, respectively (p = 0.149), while the age of mothers at parturition was 18 weeks for controls and 18.5 weeks for the treatment group. Finally, the level of water intake between control and treatment groups was measured. The water consumed during lactation is usually greater than that consumed during gestation, so these figures are presented separately. The control dams consumed an average of 5.9 ml/day during gestation and 15.6 ml/day during lactation. The treatment group consumed 6.2 ml/day of chlorite-treated water during gestation and 15.3 ml/day during lactation. The difference between treatment and control groups in the amount of water consumed was not significant for gestation period (p = 0.08) or lactation period (p = 0.87).

Effect of Chlorite Exposure on Kidney Structure

Two-way analysis of variance revealed no statistically significant treatment-related differences for any of the four physical parameters measured (body weight change, kidney weight, percent kidney to body weight ratio and weekly water consumption). Statistically significant length of exposure differences were observed in body weight change, kidney weight, and weekly water consumption; but a significant difference was absent in the percent kidney to body weight ratio (Table 3). Additionally, no significant interaction occurred between length of treatment exposures and treatment levels of any of the four parameters.

Chi-square analysis of mortality rates indicated that these rates were not significantly different between the 30-, 90- and 180-day groups nor between the five various treatment levels.

Macroscopically, the kidneys were normal. The kidneys' outer surfaces were smooth, encapsulated, and pinkish-brown in color. No external lesions or growths were observed. Internally, the kidneys were unilobar with a dark red medulla and a pinkish-red cortex. No internal lesions, growths or obstructions were observed.

Microscopic examination using light and transmission electron microscopes revealed no evidence of glomerular, vascular, obstructive or tubular disease. Glomeruli, vasculature, and tubular networks were free from significant nephritis, nephrosis, vascular sclerosis, and obstructions. Ultrastructural or minimal changes were not found.

Discussion

Based upon the data in this study and a previous one (*23*), it appears that exposure of A/J and C57L/J mice to 100 ppm of chlorite for 30 days produces increases in G6PD activity, mean corpuscular volume, osmotic fragility and acanthocytosis (regular deformations or projections on the cell surface). *In vitro* studies by Heffernan (*11,12*) revealed that incubation of human and rat erythrocytes with increasing concentrations of ClO_2^- produced a progression of alterations in the erythrocyte morphology. Concentrations of $7.4 \times 10^{-4}\,M$ to $7.4 \times 10^{-3}\,M$ ClO_2^- produced multiple sharp pointed deformations on the membrane similar to effects seen in our *in vivo* studies. *In vivo* studies by Abdel-Rahman et al. (*28*) showed morphologic changes in the erythrocytes of rats and chickens drinking CO_2, ClO_2^-, and ClO_3^- daily for 3 months. These changes were in the form of crenated spheres (echinocytes) with distortions in the middle of the erythrocytes at 100 mg/l. of ClO_2^-. The morphologic changes to erythrocytes produced by *in vivo* or *in vitro* ClO_2^- exposure appear consistent among the results of different experimenters with the *in vivo* threshold at 100 mg/l. of ClO_2^-.

The studies of both Heffernan et al. (*13*) and Abdel-Rahman et al. (*28*) demonstrated a decrease in GSH levels of erythrocytes after *in vivo* exposure of rats to chlorite. Neither the A/J or C57L mice in our study revealed a similar decrease in GSH. However, G6PD activities were elevated, indicating that in the mice adaptation may have been sufficient to prevent measurable decreases in GSH. This did not preclude damage to cell membranes which may be protected by other mechanisms.

Heffernan et al. (*13*) reported that when rats were fed sodium chlorite, the levels of GSH were the most markedly affected of the blood parameters and declined in dose-response relation to chlorite exposure, with levels of chlorite at 60 ppm causing decreases of approximately 20% in GSH levels. Additionally, decreases in hemoglobin, red cell count and packed cell volume were also evident in the study of Heffernan et al. (*13*), indicating a mild hemolytic anemia. The present studies on mice (*23*) exposed to 100 pm chlorite indicated an increase in MCV (mean corpuscular volume), osmotic fragility, G6PD activity and the number of acanthocytes. Recent *in vitro* studies by Moore et al. (*29*) have revealed that human G6PD-deficient red cells are more susceptible to chlorite-induced decreases in GSH than are cells of humans with normal G6PD activity. Future studies are needed to quantify to what extent G6PD deficients may be at risk to chlorite induced RBC alteration in normal exposure conditions.

It should be emphasized that while the activity of A/J strain was three times that G6PD activity of the C57L/J strain, a value comparable to the ratio of normal to deficient human RBC G6PD activity, the absolute comparisons are quite dissimilar. That is, the C57L/J mice (i.e., the so-called "deficient" strain) have RBC G6PD activity similar to that of normal humans even though they showed only 1/3 the activity level of the A/J strain. Thus, the C57L/J strain did not represent a true deficient in absolute terms. In fact, their ability to increase GSH levels following chlorite stress suggests the ability to adapt to oxidant stress. Likewise, unpublished investigations in our laboratory have shown that the C57L/J mice were markedly resistant to primaquine, an oxidant drug which is known to induce hemolytic anemia in G6PD deficient humans (*15*). It thus seems that the hypothesis that G6PD deficiency enhances the occurrence of chlorite-induced hemolysis in humans remains to be evaluated via a model with a deficiency comparable in absolute terms to the human deficient condition and/or epidemiologically.

Our studies of chlorite exposure on conception rate and litters of A/J mice have demonstrated that there are no effects of ingested chlorite ion (100 ppm) on pregnant dams when measuring gestation time, breeding weight or age at parturition. Consequently, these variables do not appear to confound the results. Additionally, there is no significant difference in litter size, which also rules this out as a confounding factor in influencing weaning weight and birth-weaning growth rate. Although the number of pups alive at weaning was somewhat greater for the controls, this difference again was not significant. It would be expected that fewer mice in a litter either at birth or prior to the weaning process would result in greater weight gains. However, there were fewer in the treatment groups alive at weaning, yet they weighed significantly less on the average than controls, and had less weight gain. These results appear similar to those of Shuval and Gruener (*19*) who reported that pups of dams while drinking water with nitrite (1000–3000 mg/l.) experienced increased mortality in the first three weeks and lagged behind in growth, despite having equal birth weights to controls. The conception rate of the dams drinking chlorite-treated water was reduced 17% compared to controls. These findings indicate that chlorite ion at 100 ppm concentration is capable of reducing the conception rate of A/J mice and of retarding the growth rate of A/J pups through weaning. Levels of 100 ppm chlorite represent a 10– to 100–fold increase over expected concentrations in drinking water and additional studies are being undertaken to determine minimal health effect levels. The series of sodium chlorite doses administered orally in drinking water did not result in any detectable toxic damage to the kidneys of C57L/J male mice over the 60-, 90- or 180-day exposure periods.

Environmental Protection Agency Health Effects Research Group in Cincinnati, OH, grant number R-805-993-020. Segments of this research have appeared previously (*29,30*).

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Environmental Health Perspectives
Vol. 46, pp. 39-45, 1982

Health Effects Among Newborns after Prenatal Exposure to ClO_2-Disinfected Drinking Water

By Robert W. Tuthill,* Ruth Ann Giusti,† Gary S. Moore* and Edward J. Calabrese*

Because chlorination of potable surface waters may be associated with increased risk of carcinogenicity, substitute methods for the routine disinfection of public water supplies are being explored. As part of this search, it is especially important that the potential health effects of each alternative method should be considered. Chemical treatment of drinking water by chlorine dioxide (ClO_2) is a likely alternative mode of disinfection. Two common by-products of the ClO_2 disinfection of surface water are chlorate and chlorite. These oxidants may have negative health effects on certain high risk groups. Newborns, in particular, would seem to be at increased risk to red cell damage from oxidant stress.

The historical record study being reported here compares the morbidity and mortality experience of newborns in two similar communities, one of which used chlorination and the other which used high levels of chlorine dioxide for potable water disinfection. A statistically significant positive association was found between exposure of the mother to ClO_2-treated water during pregnancy and prematurity of the newborn as assessed by the attending physician and by a greater weight loss after birth. The rates of jaundice, birth defects and fetal and neonatal mortality did not differ significantly between communities. Because of the limitations of the study design, the findings reported here should be considered suggestive rather than definitive.

Chlorine is the traditional method for disinfecting potable water supplies. However, recent studies have demonstrated that the interaction of chlorine with naturally occurring humic acids in drinking water results in the formation of trihalomethanes (*1,2*), some of which are known or suspected carcinogens (*3,4*). The most common of these trihalomethanes, chloroform, has been shown in laboratory studies to produce hepatomas in selected mouse and rat strains in the exposure range of 90-447 mg/kg of body weight (*5*). Further, a number of ecological epidemiological studies have suggested a positive association between the practice of water chlorination and cancer mortality (*6,7*), although not all similar studies have supported this association (*8*). Preliminary results of the more recent case-control studies are reported in a review by Crump et al. (*9*).

Presently, alternative disinfection methods are being actively explored as possible substitutes for chlorination of potable water supplies. Chlorine dioxide (ClO_2) disinfection is among the alternatives being considered for adoption. The oxidants chlorate and chlorite are the primary by-products resulting from ClO_2 disinfection of surface water (*4,10*). The levels of these compounds can exist in significant amounts in ClO_2-dosed water supplies although they vary with different water characteristics and treatment conditions. Chlorites may appear in concentrations of up to 50 percent of the ClO_2 demand (*10*).

There is evidence indicating that chlorates and chlorites may themselves pose a hazard to health. The oral administration of chlorates to mice in large amounts has been shown to produce methemoglobinemia with blood destruction (*11*). Richardson suggested that similar changes might develop in chronic poisoning with manifestations of anemia,

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uremia, and evidence of nephritis. Chlorite and hypochlorite are thought to oxidize hemoglobin even more rapidly than chlorate (*12*).

Heffernan et al. (*13*) exposed cats and rats to sodium chlorite in drinking water and demonstrated dose-related decreases in erythrocyte glutathione levels at chlorite concentrations as low as 50 ppm. Additionally, an increased turnover of red blood cells of cats exposed to ClO_2 suggested that a compensated hemolytic anemia had occurred. Heffernan concluded that particular attention must be paid to the exposures of individuals sensitive to hemolytic anemia.

There is evidence that the fetus *in utero* (*14*) and the newborn appear to be at increased risk to red cell damage from oxidant stress. Fetal hemoglobin is more readily oxidizible than is adult hemoglobin (*15*), and infants have a lower enzymatic capacity to reduce methemoglobin once formed (*16*). Newborns also have a flavine and vitamin E deficiency (*16*), and these substances are thought to be important antioxidant compounds. Finally, since infants consume about three times more liquid per pound of body weight than do adults (*17*), this group would be maximally exposed to the products of ClO_2 water disinfection.

The study being reported here was designed to investigate likely health effects among newborns with prenatal exposure to ClO_2-disinfected water. It was hypothesized that certain health effects might result from oxidant stress to the fetus or newborn because of the exposure of the mother to chlorate and chlorite. The hypothesis tested was that newborns with prenatal exposure to ClO_2-treated drinking water consumed by their mothers would show increased rates of jaundice, prematurity, reduced birth weight, failure to thrive and possibly higher fetal and neonatal mortality.

Methods

Since there were no water supplies in the United States known to be employing ClO_2 at high levels for disinfectant purposes, the current study was carried out using records from the 1940s. The morbidity and mortality experience of infants born in a Massachusetts community using relatively high levels of ClO_2 for water disinfection was compared to that of infants in a suitable comparison community practicing conventional chlorination of drinking water supplies.

Infants born in a one-year period following a complete year of the highest ClO_2 usage were chosen for the study to maximize the probability of observing adverse health effects should they exist. In the first step, data from birth records were linked to hospital medical records of the child and mother for both communities. Secondly, a trend analysis of fetal, neonatal, and infant mortality in the two communities was carried out to assess any mortality differences between the communities before, during and immediately following the five-year period of ClO_2 usage.

Chicopee, Massachusetts, was one of approximately 150 United States public water supplies which used chlorine dioxide for water treatment between 1944 and 1958. However, these communities generally used ClO_2 at low concentrations for taste and odor control. Chicopee initiated treatment with ClO_2 in 1945 at relatively high levels for water disinfection as well as to overcome a taste and odor problem resulting from organic and other contamination in runoff from Westover Air Force Base. Traditional chlorination had proven unsatisfactory in treating this problem.

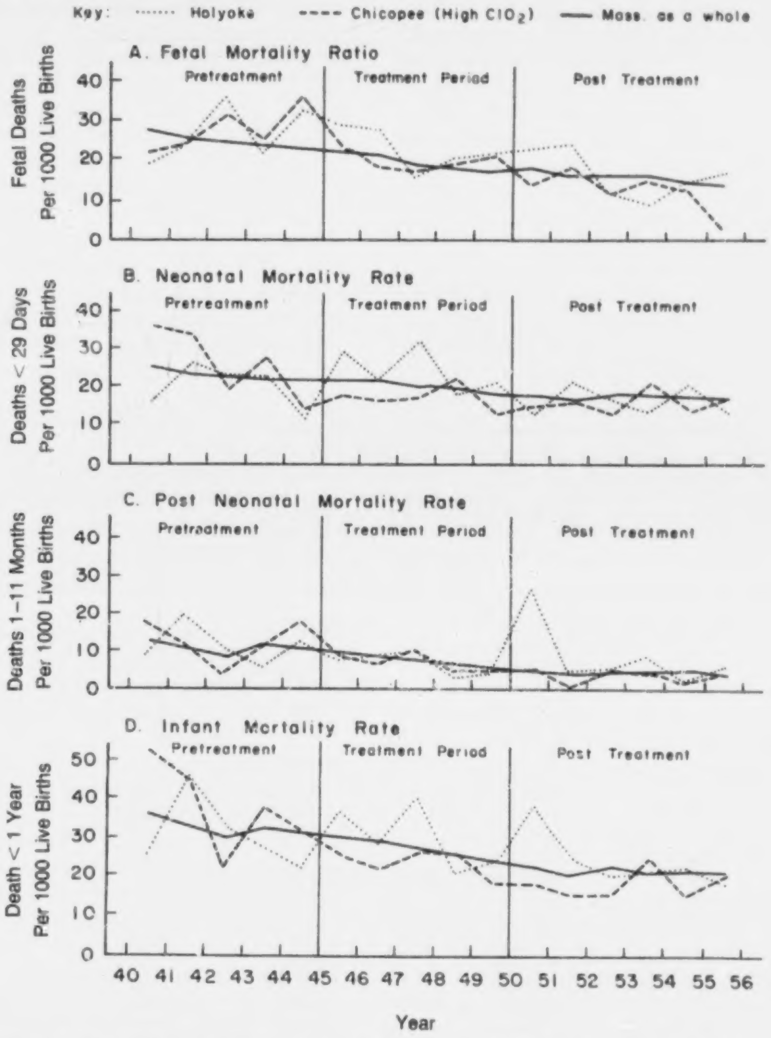
While the ClO_2 concentration formed was not directly measured, the concentration of sodium chlorite added post treatment, and the residual of chorine species, can be used as an indicator of ClO_2. As indicated in Figure 1, the highest levels of sodium chlorite were added in 1945 with a monthly high of 0.56 ppm, a monthly average of 0.32 ppm and residuals of chlorine species exceeding 0.30 ppm. Levels of sodium chlorite added declined after 1945 to levels of 0.16 ppm prior to September 1950 when the changeover was made to Quabbin, MDC water.

Holyoke, a geographically contiguous community, was selected as a comparison community. The two communities largely shared the same maternity facilities, and, as Table 1 shows, they were relatively similar in socioeconomic characteristics affecting neonatal morbidity and fetal and neonatal mortality.

Results

Neonatal Morbidity

Over 94% of the 1068 births registered to Chicopee mothers and 98% of the 1244 Holyoke births were identified as occurring in the four major hospital maternity centers. The location of births and reasons for exclusion from the study are detailed in Table 2. It should be noted that the majority of Chicopee mothers delivered at Providence Hospital or the Springfield Hospitals. For Holyoke mothers the majority of deliveries were in Providence and Holyoke hospitals. Home births, missing or destroyed records and exclusion for other reasons reduced the final study group to 903 (84.6%) of the Chicopee infants and 1112 (89.4%) of the Holyoke births. The exclusion rate was similar for the three hospital groups. It should be noted that the difference between



FIGU 1. Fetal, neonatal, postnatal and infant mortality ratio in Chicopee and Holyoke, 1940–1955. Source: Annual Vital Data Reports, Massachusetts Department of Health.

the com unities in the proportion of births at each hospita equired that all data analyses be controlled for hos tal of occurrence of the birth.

The i tial examination of the data, including the Kolmog rov-Smirnov one-sample test for normality, ind ated that the continuous study variables were fr n nonnormal population distributions. Therefore, a statistical analyses were carried out by nonpar metric methods.

The Vilcoxon stratified (partial rank sum) test was us d to assess statistically significant differences i continuous outcome and control variables betwee communities while stratifying by hospital.

Table 1. Comparison of demographic characteristics in Chicopee and Holyoke, Massachusetts, in 1950.[a]

	Chicopee	Holyoke
Total population (all ages)	49,211	54,661
Females (ages 15-44)	11,339	11,939
Nonwhite, %	0.8	0.6
Foreign-born, %	15.1	17.0
% of males 14+ engaged in manufacturing	61.9	53.0
Median income, $	3,103	2,817
Median education for persons aged 14+, years	9.1	9.9
General fertility rate	9.3	9.2

[a] Source: U.S. Bureau of the Census (*18*).

Table 2. Location of births to Chicopee and Holyoke mothers by place of occurrence in 1946 and reasons for exclusion.

	Chicopee Mothers	Hoyoke Mothers
Location of birth		
At home	29 (2.7)	15 (1.2)
Holyoke Hospital, Holyoke	81 (7.6)	481 (38.7)
Providence Hospital, Holyoke	444 (41.6)	701 (56.4)
Wesson or Mercy, Springfield	439 (41.1)	23 (1.8)
Infirmary, Springfield	31 (2.9)	1 (0.1)
Records not located[a]	44 (4.1)	23 (1.8)
Total registered births[b]	1068 (100.0)	1244 (100.0)
Source of exclusions		
Home births, no records	29 (2.7)	15 (1.2)
Records not located[a]	44 (4.1)	23 (1.8)
Records discarded[c]	31 (2.9)	1 (0.1)
Excluded for other reasons[d]	61 (5.7)	93 (7.5)
Final study sample	903 (84.6)	1112 (89.4)

[a] Hospital of birth had no medical record in their files that could be found.

[b] All registered births to Chicopee and Holyoke mothers in 1946 were accounted for.

[c] Springfield Infirmary closed down in 1947, and all medical records were subsequently discarded.

[d] Other exclusions included twins, stillborns, infants with erythroblastosis fetalis, nonwhite infants and births to Westover Air Force Base mothers who lived on the base and received a separate water supply.

Table 3. Distribution of key outcome and control variable values by hospital and community.

	City	Hospital			
		Providence	Holyoke	Wesson/Mercy	All
Number of births	Chicopee	421	79	403	903
	Holyoke	629	462	21	1112
Outcome variables					
Premature by physician assessment, %	Chicopee	10.7	6.3	1.7	7.8
	Holyoke	6.7	4.6	0.0	5.8
Birth condition listed as fair or poor, %	Chicopee	5.7	6.3	2.7	4.4
	Holyoke	5.4	3.0	0.0	4.3
Premature by birth weight, %	Chicopee	7.4	3.8	5.0	6.0
	Holyoke	6.4	6.7	0.0	6.4
Premature by gestational age (< 37 weeks), %	Chicopee	40.1	–	17.6	–
	Holyoke	41.2	–	19.1	–
Mean birth weight, g	Chicopee	3265.2	3293.7	3300.4	3283.[illegible]
	Holyoke	3318.1	3258.7	3461.8	3296.[illegible]
Mean maximum weight loss, g	Chicopee	−158.0	−157.8	−208.8	−180.[illegible]
	Holyoke	−145.1	−171.2	−208.1	−157.[illegible]
Mean weight loss at six days, g	Chicopee	−109.0	−63.4	−135.8	−117.[illegible]
	Holyoke	−93.0	−89.2	−154.0	−92.[illegible]
Control variables					
Infants formula fed, %	Chicopee	44.6	33.8	35.4	39.[illegible]
	Holyoke	52.6	43.0	23.8	48.[illegible]
Mean age of mother	Chicopee	27.1	27.1	27.2	27.[illegible]
	Holyoke	27.9	27.5	27.7	27.[illegible]
Mean vitamin K dose	Chicopee	2.73	3.21	0.85	1.[illegible]2
	Holyoke	2.74	3.31	1.33	2.[illegible]5

The dichotomous variables were analyzed using an extension of the Mantel-Haentzel procedure for summing 2 × 2 tables.

Using the above approach, there were several statistically significant differences between newborns of the two communities. As indicated in Table 3, a greater proportion of Chicopee infants were premature by physician assessment ($p < 0.05$). Although the proportion of infants defined as premature by physician assessment differed considerably by hospital, Chicopee infants were more often rated premature in all three hospitals. In concert with the assessment, a greater proportion of infants from Chicopee were rated as being in fair or poor condition at birth in all three hospitals, although the difference overall is not statistically significant.

Several other commonly used measures of prematurity did not lend consistent support to the physician assessment of prematurity. Although a statistically significantly greater proportion of Chicopee infants were premature by birth weight (<2500 g) in Providence hospital, that effect was not consistent for the other hospitals, and the overall difference, irrespective of hospital, was in the opposite direction and not statistically significantly different. Prematurity as judged by gestational age (< 37 weeks) showed a lower proportion of Chicopee infants with a shorter estimated gestational age. However, data on gestational age were not available from Holyoke hospital birth records. Finally, mean birth weight, although statistically significantly lower for Chicopee infants overall ($p<0.05$), was not consistently lower for all three hospitals.

There were no statistically significant differences between communities for the following birth outcome variables: jaundice, birth defects, discharge condition rated fair to poor, death in the first year, maximum weight loss, weight loss at six days or birth length.

Among the potential control variables, there were no statistically significant differences between the communities for the following: proportion of male births, maternal risk factors present, previous pregnancy loss, maternal gravidity, proportion lower social class, or proportion primiparous. Differences that did exist between communities were a lower percent of Chicopee infants formula fed ($p <0.05$), a lower mean ($p <0.05$) age of Chicopee mothers, and a lower vitamin K dose for Chicopee infants ($p <0.05$). In the case of the formula feeding, Providence hospital showed the greatest difference. Age of Chicopee mothers was consistently younger in all hospitals, although Providence hospital had the greatest difference. The vitamin K disparity was found primarily in the Wesson-Mercy hospitals. Social class differences between communities were found when social class was categorized into five groups ($p <0.01$), but social class was unrelated to any of the significant outcome variables of prematurity or weight change after birth.

Age of mother was both different between communities and positively related to birth weight while mode of feeding was different between communities and related to weight loss among the infants after birth. Because of the possibility of negative confounding, further analyses were carried out to examine the effects of controlling birth weight differences by age, and of controlling the weight change variables by mode of feeding. A Wilcoxon stratified (partial rank sum) test was used to assess the birth weight differences between the communities when stratifying by age of mother and by hospital. Controlling for age considerably reduced the earlier differences in mean birth weight between infants of the two communities and the differences were no longer statistically significant.

The same approach was used to assess the differences in weight change between the communities' infants when controlling for mode of feeding by the mother and for hospital site. For weight at six days compared to birth weight there was a statistically significant overall difference in mean weight loss ranks between the infants of the two communities. The difference was consistent in both hospitals for breast-fed and mixed-fed babies but there was a small difference of opposite direction between the formula-fed groups in either hospital. A similar analysis of maximum weight loss in the first six days showed an overall statistically significant difference ($p <0.01$) although there was an inconsistent direction of the association in the Holyoke-Wesson/Mercy hospital group compared to the consistently greater maximum weight loss for Chicopee infants for all feeding groups in Providence Hospital.

Infant Mortality

The mortality analysis over time showed no clear or consistent patterns of fetal, neonatal, post neonatal, or infant mortality in the ClO_2 usage community versus the comparison community. These results are detailed in Figure 2.

Discussion

Few towns, if any, in the United States employed ClO_2 as a disinfectant at the time this study was carried out. Those towns that currently use ClO_2 use it at a low level for taste and color control.

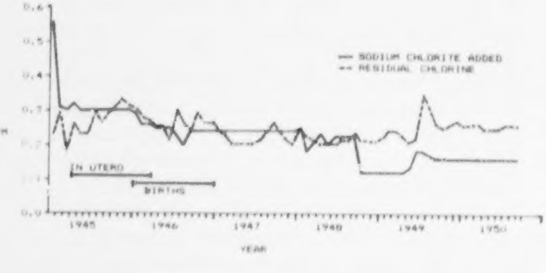


FIGURE 2. Average monthly values of $NaClO_2$ added (ppm) and residual chlorine (ppm) in Chicopee, Mass., water supply, 1945–1950. Source: chemist's reports, Chicopee Water Department.

Therefore, the methodological options for studying the health effects of ClO_2-treated drinking water on human population were limited to a historical study design of an ecological nature.

The retrospective study design imposed several limitations. Information on important control variables such as smoking, drinking, and nutritional habits of the mother during pregnancy was not available, although these habits may not have differed between communities. Additionally, the length of residence of the mother in the community could not be determined for the vast majority of test mothers. This may have resulted in inclusion of infants in the test group who were not in fact exposed to ClO_2-disinfected water during the entire length of gestation. Such a factor would have worked against finding the hypothesized relationships regarding fetal development.

An important limitation of the study resulted from the subjectivity of some of the outcome measures. The presence of jaundice, the general physical condition at birth and at discharge, and the physicians' assessment of prematurity were not clearly defined nor necessarily uniformly recorded by the physicians in 1946.

Unfortunately, the criteria for the physicians' assessment of prematurity were not specified in the records of the study hospitals, although the physicians were probably noting reflexes, muscle tone, skin appearance and other external features in making their judgments. Clearly, the physicians' assessments of prematurity were consistently higher for Chicopee infants in all three hospitals. As well, the percent of infants classified premature by the physicians did differ dramatically by hospital. The difference between communities for all hospitals combined is affected by both the big differences between hospitals as well as the differential weighting of the Holyoke and the Wesson/Mercy hospitals caused by Holyoke hospital being used predominately by Holyoke mothers and Wesson/Mercy almost exclusively by Chicopee mothers. These differences in premature assessment practices between communities could be due to differences in physicians' criteria, hospital policies, or the nature of the mothers who selectively attended that hospital. Unfortunately, the large number of attending physicians and the small number of patients seen by each physician made control for attending physician, within or between hospitals, impossible. The selective attendance problems cannot be fully assessed, although it was not related to age of mother or socioeconomic status.

Although fraught with difficulties, the study was deemed important because of the potential health implications of the proposed adoption of ClO_2 disinfection. It was felt that given the sample size and the relatively high concentration of chlorine dioxide employed in water treatment during the years studied, significant adverse health effects of prenatal exposure to treated water would be observed if they indeed existed.

Conclusions

The study found a higher percent of Chicopee newborns judged premature by physician assessment. This finding was consistent in direction for all hospitals and was unaffected by other differences between the communities. Although birth weight appeared initially lower in Chicopee infants, after controlling for the age of the mother, this difference between the communities essentially disappeared. Several other measures of prematurity likewise did not support the difference in physician assessments.

There was an indication that Chicopee infants suffered a greater maximum weight loss after birth and a smaller weight gain at six days. After controlling for mode of feeding practiced by the mother, this difference between communities remained but was not consistent in direction for all hospital/mode of feeding groups.

The limited negative health effects identified in this study were not severe or life threatening in and of themselves. However, in considering the larger significance of these findings, ClO_2 exposure should be considered as it acts in concert with other factors to produce oxygen stress. The implications of added oxidant stress from ClO_2-treated water on infants exposed to high levels of nitrites or other hemoglobin oxidizing agents, or in infants with metabolic (G6PD-deficiency) or dietary deficiencies (vitamin E) which contribute to oxidant stress could be profound. Before ClO_2 is recommended for wide use in the United States as a water disinfectant, procedures should be established to insure minimal chlorite/chlorate formation during the water treatment process. Additional studies should be undertaken to more accurately assess the potential health effects of chlorite exposure on susceptible populations before widespread use of ClO_2 is encouraged or mandated.

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Environmental Health Perspectives
Vol. 46, pp. 47-55, 1982

Subchronic Toxicity of Chlorine Dioxide and Related Compounds in Drinking Water in the Nonhuman Primate

by J. P. Bercz,* L. Jones,* L. Garner,* D. Murray,* D. A. Ludwig* and J. Boston*

Subchronic toxicities of ClO_2, $NaClO_2$, $NaClO_3$ and NH_2Cl were studied in the African Green monkeys (*Cercopithecus aethiops*). The chemicals were administered in drinking water during 30–60 days subchronic rising dose protocols. The only unexpected and significant toxic effect was elicited by ClO_2; this chemical inhibited thyroid metabolism in the animals at a dose of ca. 9.0 mg/kg/day. A statistically significant decrease of serum thyroxine occurred after the fourth week of exposure to 100 mg/l.concentration. The extent of thyroid suppression was dose dependent in each individual monkey, and was reversible after cessation of exposure. $NaClO_2$ and $NaClO_3$ failed to elicit similar effects in doses up to ca. 60 mg/kg/day. Also, $NaClO_4$ or NH_2Cl did not cause T-4 suppression in doses of 10 mg/kg/day. The selective thyroid effect of ClO_2 was unexplained and it appeared to be paradoxical since ClO_2 was rapidly reduced by the oral and gastric secretions to nonoxidizing species (presumably Cl^-). No evidence of thyroid effects were detected in the serum of human volunteers who ingested ~ 1 mg/l. of ClO_2 in drinking water as a result of routine use in the community water treatment process.

Sodium chlorite induced dose-dependent oxidative stress on hematopoesis, causing decreased hemoglobin and red cell count and increased methemoglobin content. At the same time, serum transaminase (SGPT) levels showed significant subclinical elevation. The hematologic effects of $NaClO_2$ rebounded during exposure indicating compensatory hemopoietic activity taking effect during oxidative stress. Sodium chlorate and chloramine did not induce detectable hematologic changes in the animals.

Introduction

Owing to its excellent microbicidal properties, chlorine dioxide (ClO_2), a water-soluble yellow oxidant gas, has been used in the past for drinking water disinfection. The apparent relative absence of the carcinogenic trihalomethanes (THM) in ClO_2 treated water triggered renewed interest in this compound as a possible alternative to chlorine (*1*), since the latter was shown to generate THMs (reacting) with humic substances (*2*, *3*).

Concomitantly, the toxicology of ClO_2 and its metabolites (ClO_2 and ClO_3) have received wide attention in the recent literature. By using orally administered ClO_2, hematologic changes and inhibition in testicular uptake of ^3H-thymidine was demonstrated in rats by Abdel-Rahman (*4*). Effects of these chlorine oxides on the glucose-6–phosphate dehydrogenase (G6PD)-deficient mouse were reported by Moore et al. (*5*). ClO_2 associated kinetics of red cell GSH depletion and intravascular hemolysis in rats and chickens was reported by Abdel-Rahman et al. (*6*). These workers also described the metabolism of $^{36}ClO_2$ (*7*) in rodents. In addition, the effects of ClO_2 and metabolites, as they effect the cellular GSH system in the rat, mouse and chicken blood, were investigated by Couri et al. (*8*) Oxidative *in vitro* damage to erythrocytes by $NaClO_2$ was reported

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Table 1. Clinical test protocol.

Test performed	Time of exposure, weeks						
	1	2	4	6	8	16 (Rest)	Test substance[a]
Red cell GGPD	X						1
Red cell count and indices	X	X	X	X	X		5
Reticulocytes	X	X	X	X	X	X	5
Osmotic fragility	X				X		5
Methemoglobin content	X	X	X	X	X	X	5
Hemoglobin content	X	X	X	X	X	X	5
White cell count and differential	X	X	X	X	X	X	5
Red cell GHS content	X		X		X		2, 3, 4
Creatinine and BUN	X	X	X	X	X	X	5
Total bilirubin	X	X	X	X	X	X	5
Total protein, albumin	X	X	X	X	X	X	5
Alkaline phosphatase, LDH, SGOT, SGPT	X	X	X	X	X	X	5
Total T-4	X	X		X		X	5
Body weight measurement	X	X	X	X	X	X	5

[a] Test substances: 1 = ClO_2; 2 = ClO_2^-; 3 = ClO_3^-; 4 = NH_2Cl; 5 = all.

Table 2. Hematological normal values for African Green monkeys.

Test	Sex	Normal range
Hemoglobin, g/dl	M	16.2–19.03
	F	11.85–14.72
Red cells × 10^6/mm^3	M	6.15–7.1
	F	4.57–5.85
White cells × 10^3/mm^3	M	2.72–5.52
	F	3.78–8.65
Hematocrit, %	M	49.27–59.65
	F	36.26–44.26
Reticulocytes, %	M	0.34–2.35
	F	0.14–2.06
Methemoglobin, %	M	0.0–0.75
	F	0.0–0.75

by Heffernan et al. (*9*). The same authors also examined the *in vivo* effects of $NaClO_2$, demonstrating dose dependent oxidative stress in rats (*10*).

More recently, Michael et al. (*11*) of this laboratory published results of a prospective epidemiology study, showing the absence of clinicopathological effects in human volunteers to ClO_2 treatment of their community water supply. As an adjunct to the human study, we examined the subchronic toxicity of these chlorine oxides in nonhuman primates. Additionally we incorporated monochloramine (NH_2Cl) since this chemical is an ubiquitous component of chlorinated drinking water, often purposefully generated in the water treatment process.

Materials and Methods

The Experimental Design

Each of the chemicals examined were administered to the animal colony in exponentially rising step doses, each period lasting 30–60 days depending on the availability of scheduled resources for sampling and testing the blood samples. At each dose changeover the animals were bled, followed by bi-weekly repeated bleeding. (See Table 1 for testing schedule). Between chemicals the animals were rested for 6–9 weeks, at the end of which time base lines in clinical parameters were re-established. For purposes of statistics and evaluation, each animal served as its own control.

The Animal Model

A small stable colony of African Green Monkeys (*Cercopithecus aethiops*) consisting of five adult males and seven adult females were used in the study. The body weights ranged from 3.0 to 5.7 kg, and their red cell G6PD activities ranged from 9.0–11.4 IU/g Hb with a mean of 8.8 IU/g Hb. All of the animals were in our possession for the past 8 years and repeated medical and laboratory examinations ascertained that the animals were in excellent health. Their hematological normal values are listed in Table 2. Repeated tuberculin tests and chest x-rays were performed throughout the study to assure the absence of mycobacterial infections. The animals were housed individually. They were fed a diet of Purina Monkey Chow supplemented daily with fresh fruit. During periods of exposure distilled water was made available *ad libitum*. For purposes of restraint light anaesthesia was induced by IM injection of ketamine HCl in uniform doses of 10.0 mg/kg.

Test Solutions

A stock ClO_2 solution of 400–500 mg/l. was prepared by purging ClO_2 from an acidified-$NaClO_2$

generator through an absorbent $NaClO_2$ solid column into 1 gallon quantities of distilled deionized water. The stock solution was then diluted to the appropriate concentrations (30, 100 and 200 mg/l) and was dispensed to the animals in dark bottles (2–3 liters/animal) equipped with ball-valve sipping tubes to minimize drippage. The solutions were changed, and consumption was measured three times weekly. Concentration and purity of the solutions were determined before administration and at the time of refilling to determine the extent of hydrolytic and photolytic degradation during residence in the bottle. Ultraviolet spectroscopy $E_{360_{nm}} = 1.1 \times 10^5$ $mole^{-1}cm^{-1}$) and titrimetry according to Palin (*12*) was employed for the assays. Absence of Cl_2 and of OCl^- was verified by the $AgNO_3$ test for Cl^-. All consumption measurements were made by weight differential.

Chlorite and chlorate solutions (25, 50, 100, 200 and 400 mg/l. as ionic equivalents to ClO_2) were prepared from the corresponding analytical grade sodium salts. Monochloramine solutions were prepared according to Guion (personal communication). Dispensing and dosage of these solutions were as described above.

Clinical Pathology Procedures

According to the experimental schedule shown in Table 1 blood was collected from the saphenous vein under light anaesthesia. Care was exercised not to draw more than 8 ml per bleeding period to avoid excessive blood loss and anemia.

Hematology tests (Table 1) were peformed according to standard procedures described by Wintrobe (*13*). For the determination of cell counts, cell indices and hemoglobin content a Coulter ZBI-6 cell counter and hemoglobinometer was used. Serum chemistries and enzyme activities were determined using a Union Carbide Centrifichem-400 kinetic analyzer. Red cell glucose-6-phosphate dehydrogenase (G6PD) was determined with the Calbiochem Reagent set using the Centrifichem procedure according to Favara (personal communication). Red cell glutathione (GSH) levels were determined manually by using the Biomedix DTNB reagent kit. Serum thyroxine (T-4) was determined with the SYVA enzyme amplified immunoassay (EMIT) reagent kit using the Centrifichem procedure as per SYVA's modifications. All chemistries, enzymes and T-4 determinations were made in duplicate. Quality assurance in the laboratory procedures was according to CAP standards, and quality control was achieved by using assayed and unassayed control specimen (normal, abnormal Coulter 4C, Dade Monitrol I and II).

Table 3. *In vitro* **inactivation of ClO_2 in saliva.[a]**

Saliva: ClO_2 (mg/l.)	Recovery, %	
	Spectral	Titration
1:9 (300)	39	46
1:1 (300)	26	28
1:9 (30)	12	11
1:1 (30)	5	4

[a] Reaction time = 1 min.

In Vitro Deactivation of ClO_2 by Saliva

Pooled saliva was collected by buccal scraping from anaesthetized animals. The specimen was diluted 1:5 with distilled H_2O, and the resultant solution was used for the subsequent experiment. Various ratios of ClO_2 solutions were mixed with the dilute saliva in a quartz cuvet. The absorbance of the cuvet was read in a Perkin-Elmer ultraviolet spectrophotometer at 360 nm against a distilled H_2O blank. At the same time another aliquot of the mixture was prepared and titrated according to Palin (*12*). Recovered concentrations were calculated using the molar extinction coefficient of ClO_2 of $1.1 \times 10^5 mole^{-1}cm^{-1}$, and by titer equivalent. Reactant ratios and percent recoveries are summarized in Table 3.

In Vivo Deactivation of ClO_2 in the Gastric Space

A 5.7 kg male animal was lightly sedated after an overnight fast and immobilized in a monkey-chair. The animal's stomach was intubated via the oropharyngeal route, and an aliquot of 30 ml ClO_2 (60 mg/l.) solution was instilled into the gastric space with a glass syringe. Immediately after discharging, a 15 ml aliquot of the solution was withdrawn, and its total oxidizing capacity (sum of ClO_2, ClO_2^- and part of ClO_3) was determined (within 5 min) by iodometry at pH 1.0. Of the original ClO_2 equivalent oxidizing titer, 8% was recovered after 5 min of total contact time. Because of turbidity, spectrophotometry could not be used to quantitate unchanged ClO_2.

Thyroid Function Test on Human Sera

Frozen serum samples from 350 human volunteers of the prospective epidemiology study previously reported by Michael et al. (*11*) were in our possession. Samples were selected on the basis of medical history of the volunteers. Only euthyroid

Table 4. Response of clinical tests in nonhuman primates exposure to ClO_2, $NaClO_2$, $NaClO_3$ and NH_2Cl.

Test	Response			
	ClO_2	$NaClO_2$	$NaClO_3$	NH_2Cl
Red cell G6PD	NR	NR	NR	—
Red cell count	NR	D	SID	NR
Cell indices	NR	D	SID	NR
Reticulocytes	NR	SII	NR	NR
Osmotic fragility	NR	NR	NR	—
Methemoglobin	NR	SII	NR	NR
Hemoglobin	NR	SID	SID	NR
Leukocyte count	NR	NR	NR	NR
Differential count	NR	NR	NR	NR
GSH red cell	—	—	NR	NR
Creatinne and BUN	NR	NR	NR	NR
Total bilirubin	NR	NR	NR	NR
Total protein, albumin	NR	NR	NR	NR
Alkaline phosphatase, LDH	NR	NR	NR	NR
SGOT	NR	NR	NR	NR
SGPT	NR	I	NR	NR
Serum T-4	D	SID	NR	NR
Body weight	NR	NR	NR	NR

[a] Response legend: NR = no response ($p < 0.01$); D = statistically significant decrease with dose dependence; I = statistically significant increase with dose dependence; SII = slight increase with dose dependence; SID = slight decrease with dose dependence; — = not done.

Table 5. Effects of ClO_2, $NaClO_2$, $NaClO_3$ and NH_2Cl on circulating total thyroxine in primates.

Test chemical	Serum T-4, μg/dl					
	0	30 mg/l. (4 wk)	100 mg/l. (1 wk)	100 mg/l. (6 wk)	400 mg/l. (8 wk)	Rest
ClO_2	4.7	4.90[a]	4.4	3.5[b]	—	5.3
$NaClO_2$	4.99				4.43[c]	5.3
$NaClO_3$	5.3				5.7[d]	5.4
NHCl	5.28			5.8[e]		

Range of CV-s (Between Animals): 17 – 27%
Range of Precision (Between runs, Date Monitrol I Assayed Control Serum) 13.6% $\bar{X} = 5.66 \pm 0.77$
Quoted Range: 4.2 – 6.4

[a] Total body dose = 3.5 ± 0.9 mg/kg/day.
[b] Total body dose = 9.5 ± 5.8 mg/kg/day.
[c] Total body dose = 58.4 ± 27.6 mg/kg/day.
[d] Total body dose = 54.2 ± 38 mg/kg/day.
[e] Total body dose = 10 mg/kg/day.

Table 6. Thyroid function in human population exposed to ClO_2 in drinking water.[a]

	N	Serum T-4, meq/dl[b]
Exposed group, March	115	7.59 ± 2.2
Exposed group, June	111	7.8 ± 2.4
Control group, March	79	7.67 ± 2.03
Control group, June	72	7.7 ± 2.2

[a] Only euthyroid volunteers included.
[b] As $\bar{X}$ ± SD.

individuals were included in the study. Thyroid status of individuals with known thyroidopathies were also confirmed. The method for thyroxine determination was identical to that used for the nonhuman primate samples.

Results

Table 4 lists all clinical tests with annotations dose responses to each chemical tested.

The thyroid hormone measurement results obtain with the monkey specimen are listed in Table 5, a the human T-4 values are summarized in Table The oral doses of the four chemicals tested are al summarized in Table 6.

Figure 1 is a graphic representation of the th roid inhibition regression in twelve animals versu mean doses of ClO_2 ingested. Each point signifie by an X on the graph represents the X/Y coordinat of the thyroxine shift versus the mean dose ingeste by a single animal during the 4-week exposure t 100 mg/l. of ClO_2.

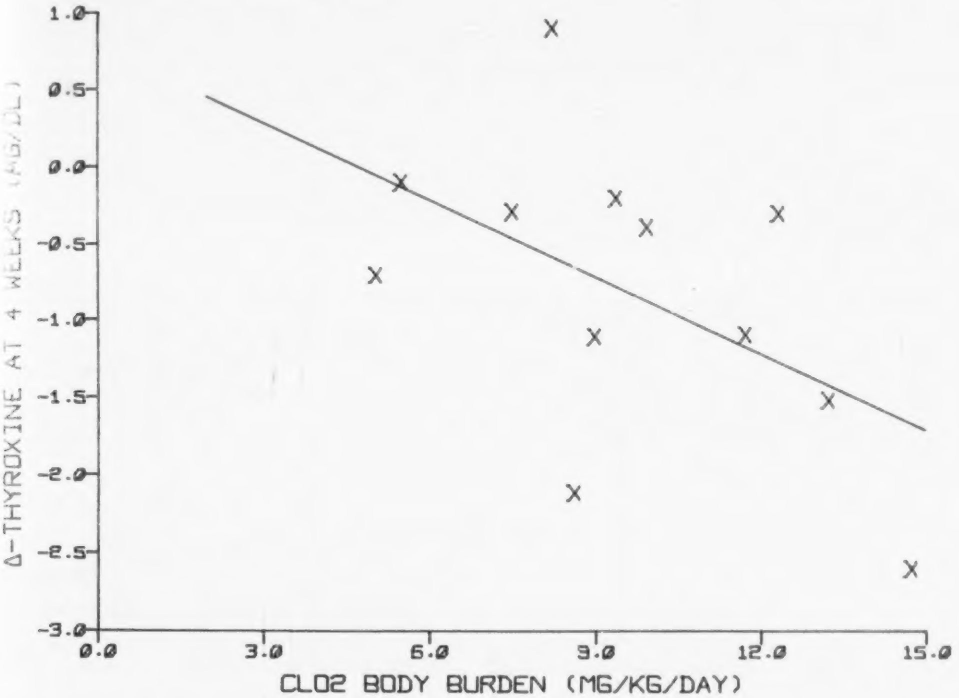

FIGURE 1. Correlation between thyroid depression and ClO_2 consumption at 4th week exposure to 100 ppm. Slope = 1.47; y intercept = 0.86; $r = 0.53$; $t = 1.98$; $t(0.05) = 1.79$.

Figures 2 and 3 depict the chronological changes in hematologic parameters in male and female monkeys during $NaClO_2$ exposure. Figures 4 and 5 represent the temporal behavior of the hematologic values during $NaClO_3$ exposure.

Table 7 lists the mean SGPT and SGOT enzyme activities in 12 monkeys during the step dose $NaClO_2$ administration.

Discussion

In view of the extent of hematologic oxidative effects and causative concentrations ranging up to 1000 mg/l. in rats and in other animals reported by Abdel-Rahman (*4*) and Moore (*5*), the apparent absence of hematologic and clinicochemical effects in the monkeys is not surprising. At the higher concentration stages of this study (100 and 200 mg/l.) the mean daily dose remained nearly constant at ca. 9 mg/kg/day. This observation reflects on the strong irritating nature of ClO_2 solutions. During the 200 mg/l. exposure, erythema and ulceration of the oral mucosa, mucous nasal discharge and avoidance of drinking water by the animals was observed. Throughout the ClO_2 study liquid consumption decreased with the strength of the solution. For example, at 0 mg/l. the mean water consumption was ca. 125 ml/kg/day with some seasonal fluctuations; at 100 mg/l. the consumption decreased to ca. 95 ml/kg/day, which further decreased to ca. 55 ml during the 200 mg/l. concentration. In fact, the high dose study was terminated after one week because some of the animals showed signs of dehydration and azotemia.

The most striking effect of ClO_2 was on the thyroid gland. At the ca. 9 mg/kg/day dose this chemical appeared to be a potent inhibitor of thyroid synthesis (Table 5). Analysis of the water consumption data at the 100 mg/l. exposure disclosed that the T-4 depression in each animal was statistically related to water consumption and ClO_2 dose (Fig. 1). Since the animals' fluid intake was near normal, the effect cannot be explained by dehydration. Adverse influence of ketamine-nitrous oxide anaesthesia on thyroid metabolism in human patients was published by Matsuki et al. (*14*). The possibility of ketamine-induced thyroid inhibition, however, must be discounted, since we followed identical ketamine anaesthesia protocols during each study of the four chemicals. One may also propose that ClO_2^- and/or ClO_3^-, which were shown to be metabolites of ClO_2 (*6*), could inhibit iodine metabo-

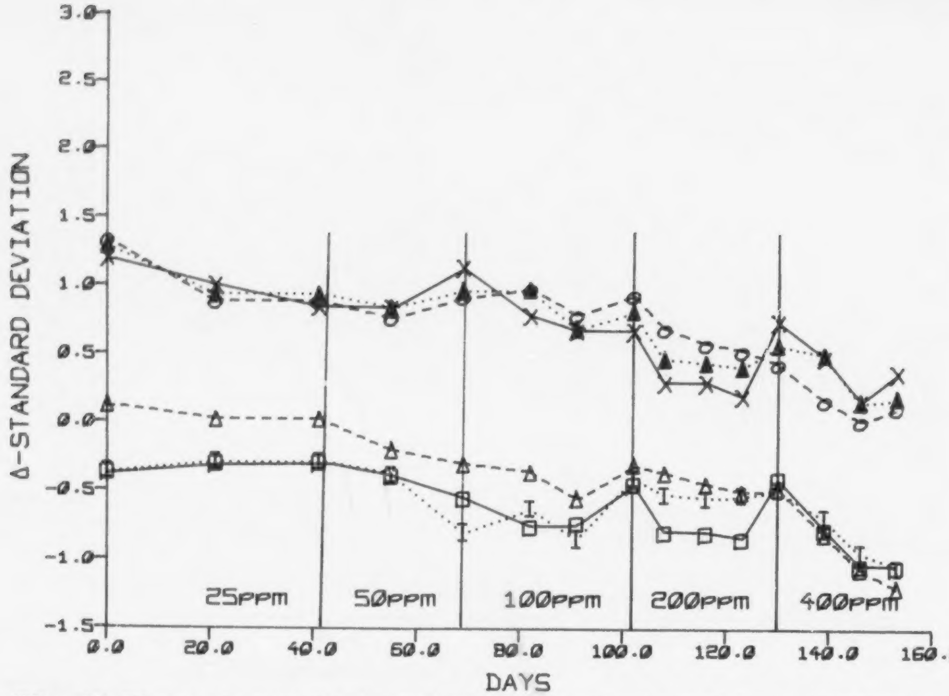

FIGURE 2. Effect of $NaClO_2$ on erythropoiesis of monkeys: (X) Hb males; (□) Hb females; (○) RBC males; (Δ) RBC females; (▲) Hct males; (I) Hct females.

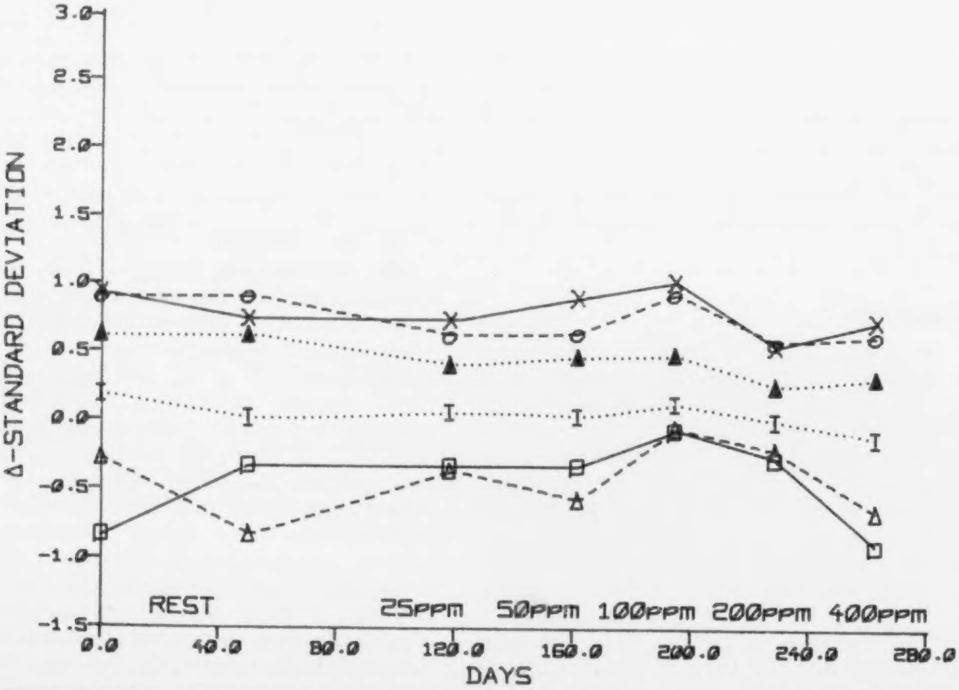

FIGURE 3. Effect of $NaClO_2$ on methemoglobinemia in monkeys: (X) reticulocytes males; (□) reticulocytes females; (○) methemoglobin males; (Δ) methemoglobin females.

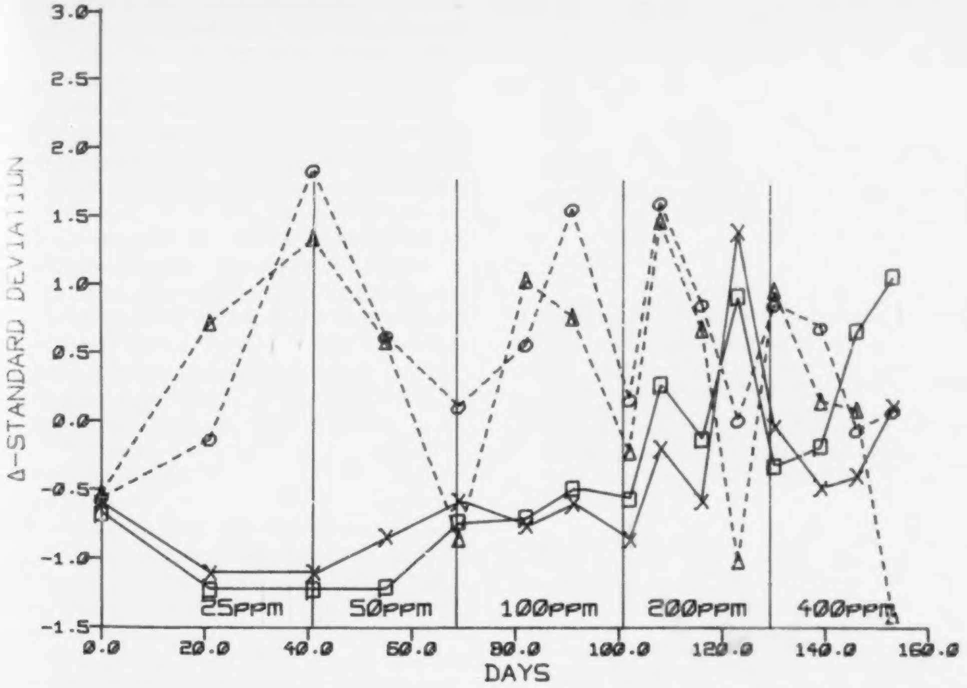

FIGURE 4. Effect of $NaClO_3$ on erythropoiesis in monkeys: (X) Hb males; (□) Hb females; (○) RBC males; (Δ) RBC females (▲) Hct males; (I) Hct females.

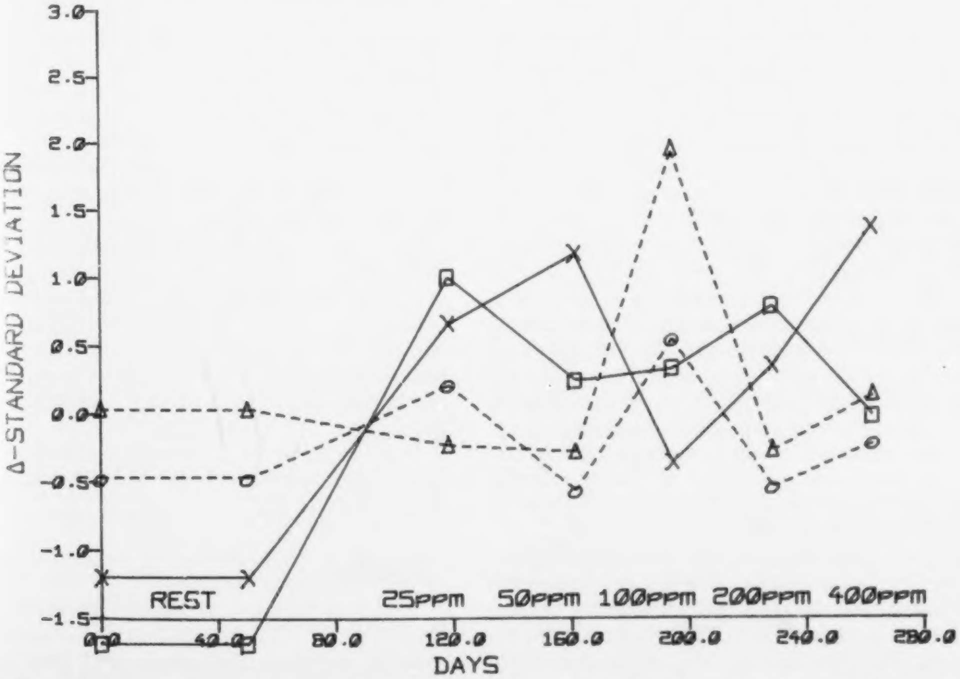

FIGURE 5. Effect of $NaClO_3$ on methemogloginemia in monkeys: (X) reticulocytes males; (□) reticulocytes females; (○) methemoglobin males; (Δ) methemoglobin females.

Table 7. Mean AST and ALT activities in monkey serum during $NaClO_2$ exposure.

Dose, ppm	N	AST, IU/l.	ALT, IU/l.
25	12	22.3	4.1
50	12	24.46	5.7
100	12	24.75	6.4
200	12	25.35	19.8
400	12	23.00	20.0

[a] $r^2 = 0.78$; $t = 3.28$; $t\,(0.05) = 2.35$.

lism in analogous fashion to the known goitrogenic perchlorate ion, (*15*, *16*) as shown in Eqs. (1)-(3).

$$ClO_2 + e^- \xrightarrow[\text{reduction}]{\text{Metabolism}} ClO_2^- \tag{1}$$

$$2\,ClO_2 + H_2O \xrightarrow[\text{hydrolysis}]{\text{Metabolism}} ClO_2^- + ClO_3^- \tag{2}$$

$$ClO_2^-/ClO_3^- \xrightarrow{\text{Absorption}} \text{Inhibition of } I^- \text{ metabolism within the thyroglobulin, analogous to } ClO_4^-. \tag{3}$$

This hypothesis is invalid because $NaClO_2$ and $NaClO_3$ failed to elicit thyroid inhibition in doses up to 60 mg/kg/day. Moreover, $NaClO_4$ administered in drinking water did not show antithyroid effects in 10 and 20 mg/kg/day doses during a 30–day pilot trial. Perhaps the strongest argument against the above hypothesis arises from our *in vivo* recovery study, which demonstrated that ingested ClO_2 is rapidly reduced in the acidic stomach juices to nonoxidizing species (probably Cl^-). We were able to recover only 8% of the total oxidizing capacity equivalent of the ClO_2, instilled into the animal stomach, after 5 min. of contact time with the gastric contents. This finding is in partial agreement with the radiolabeling studies of Abdel-Rahman et al. (*6*) that demonstrated approximately 80% of the radiochlorine in plasma was in the Cl^- form and about 20% of the $^{36}ClO_2$ was metabolized to ClO_2^- in the rat. A similar distribution of radiochlorine in Abdel-Rahman's study was found in the urine of the animals.

Based on these considerations, it is unlikely that the absorption of a simple chlorine oxide species caused the thyroid effects. An alternative mechanism may be that decrease of dietary iodine absorption in the GI tract due to ClO_2 induced mucosal pathology. Abdel-Rahman demonstrated in rats the distribution of radiochlorine after $^{36}ClO_2$ administration was prominently high in the stomach and intestines 72 hr post-administration, indicating the halogen may be covalently attached to the mucosal surface. Inhibition of iodine absorption and alteration of the bioavailability of dietary iodine in the injured intestinal tract would result in progressive iodine deficiency. This was supported by the observation that the T-4 deficiency developed slowly (4 weeks) and progressively in the animals.

Another possibility is that chemical reactions between nutrients and ClO_2 give rise to thyroid inhibitory molecules in the GI tract. Such products may interfere with iodine uptake in the thyroglobulin, or they could displace T-4 from binding sites of the carrier, thyroxine-binding globulin. Past toxicologic studies with food ingredients treated with ClO_2 (e.g., bleached flour) disclosed no adverse effects on rabbits, monkeys (*17*) and rats (*17*), and on dogs (*17*, *19*). Moran et al. (*20*) identified several modified amino acids in wheat gluten treated with ClO_2, e.g., methionine sulfone and mono- and dichlorothyrosine. Other chemical effects were also seen, such as decrease of tocopherol content, etc. Considering the chronology of these studies and the unavailability of methods for thyroid assessment at the time, effects of the ClO_2 modified nutrients on thyroid metabolism, if they existed, would have been missed. From Abdel-Rahman's work an additional possibility emerges. About 25% of the radiolabeled chlorine in the liver of rats administered $^{36}ClO_2$ is bound to proteins in the cellular sap, indicating the radiochlorine species may be bound by covalent or by strong hydrogen bonding to these proteins. Such findings could be explained by incorporation of chlorinated amino acids in the protein matrix, or conversely involvement of chlorinated amino acids in thyroid metabolism could be a possible explanation for the observed effect.

Our attempt to find thyroid effects in the serum of human volunteers was unsuccessful. This group consumed only about 1 mg/l. of ClO_2 for every liter of drinking water, in which the ClO_2^- and ClO_3^- content did not exceed 5 mg/l. (*11*). The estimated human dose of ClO_2 in this study was only about one thousandth (1×10^{-3}) of that administered to the monkeys.

Further research is in progress to determine the nature and mechanism of the thyroid inhibitory effect of ClO_2.

Chlorite

Previous findings of Heffernan et al. (*10*) and Moore et al. (*5*) demonstrating oxidative stress induced methemoglobinemia and anemia in rats was verified in this study. Our results show that at most doses ClO_2^- induced a self-compensating oxidative stress in the monkey hematopoiesis. About midway through exposure, a rebound phenomenon occurred in hemoglobin and red cell synthesis. Figures 2 and 3 show male and female hematologic parameters plotted in terms of Delta Standard Deviations (ΔSD) for comparability. The reticulocytotic response (Fig. 3, solid lines) to ClO_2^- exposure was not accompanied by dose responsive methemoglobinemia.

During $NaClO_2$ exposure a dose-dependent rise of ALT was detected in the monkeys. The extent of enzyme evaluation was subclinical (Table 7) and was not corroborated by concomitant elevation of any other enzyme system or by serum bilirubin. The significance of this observation is not known, but may be associated with accelerated hepatic activity during the transient oxidative hemolytic period.

Chlorate

The effects of chlorate were similar to those seen during chlorite exposure (Fig. 4 and 5), although the rebound phenomenon was not as clearly discernible.

Chloramine

At the attainable maximum concentration (100 mg/l.) this compound appeared to have no detectable effect on the hematology of the animals, including red cell GSH content. No evidence of thyroid suppression was detected in the serum.

Conclusions

The thyroid inhibitory effects of ClO_2 ingestion appears to be a significant health endpoint of unknown explanation. The potential for adverse health effects during long-term chronic exposure to low levels of ClO_2, specifically to ClO_2–modified nutrients, merits further research.

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Environmental Health Perspectives
Vol. 46, pp. 57-62, 1982

Controlled Clinical Evaluations of Chlorine Dioxide, Chlorite and Chlorate in Man

by Judith R. Lubbers,* Sudha Chauan,* and Joseph R. Bianchine*

To assess the relative safety of chronically administered chlorine water disinfectants in man, a controlled study was undertaken. The clinical evaluation was conducted in the three phases common to investigational drug studies. Phase I, a rising does tolerance investigation, examined the acute effects of progressively increasing single doses of chlorine disinfectants to normal healthy adult male volunteers. Phase II considered the impact on normal subjects of daily ingestion of the disinfectants at a concentration of 5 mg/l. for twelve consecutive weeks. Persons with a low level of glucose-6-phosphate dehydrogenase may be expected to be especially susceptible to oxidative stress; therefore, in Phase III, chlorite at a concentration of 5 mg/l. was administered daily for twelve consecutive weeks to a small group of potentially at-risk glucose-6-phosphate dehydrogenase-deficient subjects. Physiological impact was assessed by evaluation of a battery of qualitative and quantitative tests. The three phases of this controlled double-blind clinical evaluation of chlorine dioxide and its potential metabolites in human male volunteer subjects were completed uneventfully. There were no obvious undesirable clinical sequellae noted by any of the participating subjects or by the observing medical team. In several cases, statistically significant trends in certain biochemical or physiological parameters were associated with treatment; however, none of these trends was judged to have physiological consequence. One cannot rule out the possibility that, over a longer treatment period, these trends might indeed achieve proportions of clinical importance. However, by the absence of detrimental physiological responses within the limits of the study, the relative safety of oral ingestion of chlorine dioxide and its metabolites, chlorite and chlorate, was demonstrated.

Introduction

Chlorine dioxide is currently under serious consideration in the United States as an alternative to chlorine water treatment. Before chlorine dioxide may be used routinely as a water disinfectant, the safety of oral human ingestion of chlorine dioxide and its by-products must be assessed. For this purpose, a controlled clinical evaluation of chlorine dioxide, chlorite and chlorate was undertaken under the auspices of USEPA HERL #CR805643.

The study was conducted in three parts. Phase I was designed to evaluate the acute physiological effects of progressively increasing doses of disinfectants administered to normal healthy adult males. Chronic ingestion by normal male volunteers was studied in Phase II. Phase III assessed the physiological response of a small group of potentially susceptible individuals, those deficient in glucose-6-phosphate dehydrogenase, to chronic ingestion of chlorite.

Methods

Subject Selection

For Phase I and for Phase II, normal healthy adult male volunteers were selected. No prospective study participant who exhibited a significant abnormality in the routine clinical serum analysis,

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blood count, urinalysis, or electrocardiogram was selected. Subjects manifested no physical abnormalities at the pretreatment examination, were 21 to 35 years of age, and weighed within ± 10% of normal body weight for their frame and stature. A history of disease or any medical or surgical condition which might interfere with the absorption, excretion, or metabolism of substances by the body precluded inclusion. Regular drug intake prior to the start of the investigation, either therapeutic or recreational, resulted in exclusion from the study. Normal methemoglobin levels, thyroid function, and glutathione levels were mandatory. Written informed consent was obtained from each subject prior to initiation of treatment.

For Phase III, volunteers were defined as glucose-6-phosphate dehydrogenase (G-6-PD)-deficient on the basis of a hemoglobin G-6-PD level of less than 5.0 IU/GM hemoglobin in the pre-study screening. Phase III subjects were normal in all other respects.

Water Disinfectant Preparation

A detailed description of the water disinfectant preparation techniques has been presented by Lubbers and Bianchine (*1*). In general, freshly prepared stock solutions of chlorine dioxide, sodium chlorite, sodium chlorate, chlorine and chloramine were assayed by the colorimetric techniques of Palin (*2*) then diluted with organic-free demineralized deionized water to appropriate concentrations. Individual bottles were capped and stored in the dark under refrigeration until use. All bottles were coded by an independent observer and the identity of each bottle remained "double-blind" to both the investigative staff and the volunteer subjects.

Study Design: Phase I

The 60 volunteers in Phase I were divided at random into six treatment groups (*1*). Ten persons were assigned to receive each of the disinfectants; the ten members of the control group received untreated water. The study involved a series of six sequences of three days each. Treatment concentrations were increased for each treatment. The specific concentrations or disinfectant administered to the study participants are listed in Table 1. A clinical evaluation of the collection of blood and urine samples for determination of pretreatment baseline laboratory values preceded the first treatment. On the first day of each three day treatment sequence, each volunteer ingested 1000 ml of the water in two portions. The second 500 ml portion aliquot was administered 4 hr after the first. Each 500 ml portion was consumed within 15 min. Only two doses of disinfectant were administered on the first day of each treatment sequence. No disinfectant was administered on the second and third day of each sequence, since these two days were to serve as followup observation days. The second day of the treatment sequence consisted of a physical examination and collection of blood and urine samples for determination of posttreatment laboratory values. On the third day, each volunteer was given a physical examination to determine residual effects of treatment with the water disinfectant and byproducts.

Taste evaluations were obtained at each dose level. Study participants were asked to rate the treated water as very unpleasant, slightly unpleasant, not pleasant, pleasant, or tasteless.

Study Design: Phase II

The sixty volunteers of Phase II were divided at random into six treatment groups of ten subjects each (*3*). In order to assure efficient management of the 60 subjects, they were randomly assigned to three subsets. These subsets were sequentially entered into the study on three successive days and exited from this study in a similar fashion. For all of the treatment groups, the concentration of disinfectants ingested was 5 mg/l. The control group received untreated water. Each subject received 500 ml daily for 12 weeks. Physicals, collection of blood and urine samples for laboratory assays, and taste evaluations were conducted on a weekly basis during the treatment period and for 8 weeks following cessation of treatment.

Study Design: Phase III

The three glucose-6-phosphate dehydrogenase-deficient subjects of Phase III were given sodium chlorite at a concentration of 5 mg/l. chlorite (4). The treatment protocol was identical to that of Phase II, with daily administration of 500 ml of solution to each volunteer.

Evaluation Procedures

An extensive battery of parameters was monitored to assess the biochemical and physiological response to the oral ingestion of the water disinfectants and water treatment by-products (Table 2). All laboratory determinations of biochemical parameters were conducted by a licensed medical laboratory, Consolidated Biomedical Laboratories, Inc. (CBL), Columbus, Ohio, HEW license number 34–1030. For each volunteer, pretreatment baseline values and six sets of posttreatment values were compiled. Laboratory tests were carefully chosen.

Table 1. **Concentration of disinfectants in phase I: acute rising dose tolerance.**[a]

	Disinfectant concentration, mg/l.					
Water disinfectant	Day 1	Day 4	Day 7	Day 10	Day 13	Day 16
Chlorate	0.01	0.1	0.5	1.0	1.8	2.4
Water control	0	0	0	0	0	0
Chlorine dioxide	0.1	1.0	5.0	10.0	18.0	24.0
Chlorite	0.01	0.1	0.5	1.0	1.8	2.4
Chlorine	0.1	1.0	5.0	10.0	18.0	24.0
Chloramine	0.01	1.0	8.0	18.0	24.0	

[a] For each dose, two portions of 500 ml each were administered at 4-hr intervals.

Table 2. Biochemical parameters assayed in the controlled clinical evaluation of chlorine dioxide, chlorite and chlorate in man.

Serum chemistry	Plasma glucose, sodium, potassium, chloride, urea nitrogen, creatinine, BUN/creatinine ratio, uric acid, calcium, phosphorus, alkaline phosphatase, gamma glutamyl transferase, total bilirubin, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, lactic dehydrogenase, cholesterol, triglycerides, total protein albumin, globulin, albumin/globulin ratio, iron
Blood count	Platelet count, white blood cell count, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, high peroxidase activity, neutrophils, lymphocytes, monocytes, eosinophils, basophils, large unstained cells
Urinalysis	Color,[a] appearance,[a] specific gravity, pH, protein, sugar,[a] acetone, blood,[a] white blood count, red blood count, casts,[a] crystals,[a] bacteria,[a] mucus[a], amorphous cells,[a] epithelial cells
Special tests	Serum haptoglobin, sickle cell,[a] methemoglobin, glucose-6-phosphate dehydrogenase, Coombs test,[a] hemoglobin electrophoresis,[a] T-3 (uptake), T-4 (RIA), free thyroxine index, electrocardiogram[a]
Physical exam	Systolic blood pressure, diastolic blood pressure, respiration rate, pulse rate, oral temperature

[a] These parameters yielded qualitative data only; no statistical analysis was performed.

On the basis of the literature (*5–8*), areas of suspected biochemical response to ingestion of chlorine oxidants were defined; a portion of the test battery was specifically devoted to monitoring this response. Red blood cell surface antibody formation was clinically monitored by the qualitative Coombs test; thyroid function by T-3 (uptake), T-4 (RIA), and free thyroxine; and response to oxidative stress by glucose-6-phosphate dehydrogenase, methemoglobin and glutathione levels. Hemoglobin electrophoresis was used to detect possible hemoglobin abnormalities. A battery of peripheral parameters was assayed to provide supplementary information and to assist in evaluation of overall physiological well-being. The specific serum, blood and urine parameters assayed have been discussed by Lubbers and Bianchine (*1*).

The numerical values obtained were collected and analyzed by utilizing the facilities of The Ohio State University Division of Computing Services for Medical Education and Research. Specially designed programs facilitated rapid clinical feedback. Any value for an individual subject which differed from the group mean by more than two standard deviations was noted. In addition, every individual value which fell outside normal laboratory ranges for that parameter was designated as abnormal. Chemical parameters for volunteers who exhibited abnormal values were subjected to careful scrutiny; the safety and the possibility of hypersensitivity to the disinfectant agents were evaluated for each of these individuals on a continuing basis throughout the study.

Statistical analyses utilized commercially available computer packages, specifically, the Biomedical Computer Programs (BMDP) and the Statistical Package for the Social Sciences (SPSS). Two-way analyses of variance with repeated measures utilized BMDP2V. BMDP1R was used to perform multiple linear regression analyses. For pairwise *t*-tests and simple *t*-tests, SPSS-*T*-test was employed.

Results

Qualitative

An important aspect of this study was the careful and continued medical observation of all subjects. The general clinical histories and physical examinations alone with subjective observations and qualitative laboratory tests throughout this study were accumulated in each subject's medical file. A careful inspection of each of these medical files presented a review of the general clinical health of each subject.

The careful clinical evaluation of every subject in Phases I, II and III failed to reveal any clinically important impact upon the medical well-being of any subject as a result of disinfectant ingestion. Further, there was no apparent grouping of the minor subjective symptoms and objective signs noted throughout the study; the "colds," "lymphadenopathy," "sore throats" and "flu" problems noted episodically appear to be randomly dispersed among the treatment groups. All subjects remained negative with respect to the Coombs tests and the sickle cell tests during the investigation. Hemoglobin electrophoresis results indicated that, in Phase II, a small number of subjects yielded abnormal hemoglobin distributions but these individuals were found to be randomly distributed in both the treatment groups and in the control group. Examination of electrocardiograms revealed no abnormalities.

Vital signs (blood pressure, pulse rate, respiration rate and body temperature) were measured on a regular basis to provide immediate feedback to the monitoring physician on the acute physiological response of study participants to treatment. The statistical analysis of the vital signs was limited to the calculation of arithmetic group means and standard deviations from the mean. The compiled vital signs were examined for evidence of consistent response to treatment. No such evidence was found.

The subjective evaluations of palatability indicated that few subjects found the test substances to have an objectionable taste at levels up to 24 mg/l.

Quantitative

For the Phase I acute rising-dose tolerance study, a two-way analysis of variance with repeated measures was used to compare the treatment group values of each biochemical parameter to the corresponding values of the control group. The analysis of variance allowed distinctions to be made among the possible sources of variation. Differences between two groups that existed prior to treatment, parallel variations in quantitative chemical values due to laboratory drift and authentic treatment-related changes in physiological parameters could be distinguished. Three probabilities were calculated for each case: the group main effect (G), the time main effect (R), and group-time interaction (RG). The treatment groups and the corresponding biochemical parameters for which a strong probability of treatment-related change was computed (that is, $RG \leq 0.05$) are listed in the first column of Table 3.

To assist in determining the clinical importance of the statistically significant group time interactions, the group, mean and standard deviations from the mean were examined for the pretreatment baseline assay and each posttreatment assay for each of the treatment groups. In all instances, the group mean values remain well within the established normal ranges.

On the basis of the small magnitude of change within the normal range and the duration of the study, it was concluded that the trends identified by the analysis of variance are unlikely to be of clinical importance. The possibility that the trends might become clinically important with increased exposure cannot be excluded.

Alternative statistical techniques were employed for Phase II. An omnibus testing technique was used initially. To test the hypothesis that the response of one or more of the groups was different to that of the rest of the groups, an analysis of variance with repeated measures was performed in which values for all six treatment groups were included. For the parameters urea nitrogen and mean corpuscular hemoglobin, RG-values < 0.05 were obtained. Supplementary tests were performed. Analyses of variance with repeated measures in which the values of each treatment group were compared to the corresponding values of the control group were chosen. The use of the analysis of variance in this manner is flawed by the common control group. However, the results of the analyses may be used with caution. The analysis of variance yielded statistically significant RG-values in the comparison of the group mean corpuscular hemoglobin values for the chlorite and the chlorate groups and of the group mean urea nitrogen values of chlorate and chlorine dioxide treatment groups to the corresponding control group values, as shown in Table 3.

No linear trends were detected by linear regression analysis of the chlorite group's mean corpuscular hemoglobin values, the chlorate group's urea nitrogen levels or the chlorite group's urea nitrogen values.

Mean corpuscular hemoglobin levels in the chlorate group yielded a probability of 0.01 upon linear regression analysis. The relative slope associated with the change during the 12-week treatment period was approximately 1% of the normal physiological range per week. We believe that no physiological importance may be attributed with confidence to the variation. However, it is impossible on the basis of this study to rule out the potential physiological significance of the trend. Further study is warranted.

The small number of subjects (three) in Phase III negated the value of many statistical procedures. Linear regression analyses were chosen. The third column of Table 3 lists the biochemical parameters for which a high probability of change with respect to time was calculated. The *p*-values computed by

Table 3. Biochemical parameters and treatment groups in which statistical analyses indicated a high probability of change which could be attributed to ingestion of disinfectant.

Test	Phase I[a]	Phase II[b]	Phase III[c]
Urea nitrogen (BUN)	Chlorite	Chlorate Chlorine dioxide	
Creatinine	Chlorite Chlorine		
BUN/creatinine Ratio	Chlorite		
Uric acid	Chlorine dioxide		
Calcium	Chlorine		
Gamma glutamyl transferase	Chlorine		
Total bilirubin	Chlorate		
Albumin/globulin ratio			Chlorite
Iron	Chlorate		
Methemoglobin	Chlorate		Chlorite
T-4 (RIA)			Chlorite
Free thyroxine index			Chlorite
Mean corpuscular hemoglobin		Chlorite Chlorate	
Mean corpuscular hemoglobin concentration			Chlorite
Lymphocytes	Chlorine		

[a] Two-way analysis of variance yielded group-time interactions (RG values) ≤ 0.05 in comparisons of treatment group values to those of the control group.

[b] Two-way analysis of variance yielded group-time interactions (RG-values) ≤ 0.05 in both the omnibus and treatment group-control group comparisons.

[c] Linear regression analysis indicated a strong probability of change with respect to time; *p*-values ≤ 0.05.

the linear regression analysis were less than 0.05 for four biochemical parameters. To gauge the relative magnitude of change, the percent change of the normal range per week was computed. These statistical analyses indicate a good probability that, for A/G ratio, T-4 (RIA), free thyroxine, mean corpuscular hemoglobin concentration, and methemoglobin values, a change with respect to time occurs during the 12-week treatment period. However, in the absence of a concurrent control group and taking into consideration the small group size and the possibility of laboratory drift, one must exercise caution in dealing with the results. We can say with confidence only that trends were indicated. We cannot say that these trends were of physiological origin nor can we attribute physiological consequence to them.

Discussion

Several researchers have addressed the physiological effects of oral ingestion of the oxidizing agents, chlorine dioxide, chlorite and chlorate. Musil et al. (*9*) associated oral chlorite ingestion with methemoglobin formation. In studies by Heffernan et al. (*7,8*), Abdel-Rahman et al. (*5*) and Couri et al. (*6*), hemolytic anemia and suppressed glutathione levels were observed in animals treated with chlorite. The oral administration of chlorate to laboratory animals has been shown to induce oxidative destruction of hemoglobin and methemoglobin formation (*10, 11*). The possibility of renal toxicity at high levels of chlorite ingestion was suggested by the increased kidney/body weight ratio reported by Heffernan et al. (*7*). Haller and Northgraves (*12*) and Fridlyand and Kagan (*13*) examined the chronic toxicity of orally consumed chlorine dioxide in rats; a slightly increased two-year mortality rate and a decreased rate of weight gain were observed. Oral administration of chlorite (*14–16*) to mice was shown to increase mean corpuscular volume, osmotic fragility, and glucose-6-phosphate dehydrogenase activity of erythrocytes; morphologic changes were reported. In the African Green monkey, chlorine dioxide adversely affected thyroid function; chlorite ingestion yielded transient changes in hemoglobin levels and red cell count (*17*). The maternal toxicity, embryonic toxicity and the teratogenic potential of concentrations of sodium chlorite was evaluated in rats (*18*).

Unfortunately, the information available on the impact of chlorine dioxide, chlorite, and chlorate ingestion in man is severely limited. Epidemiological studies (*19,20*) have failed to conclusively identify any significant exposure related effects. The clinical evaluation described in this report was an attempt to elucidate the effects of the chlorite, chlorine dioxide and chlorate in man under controlled clinical conditions.

During the course of the three-phase study, a massive volume of raw data was acquired. Routine urinalyses were performed and a meticulous examination of this body of information was made. No definitive finding of detrimental physiological impact was made in any of the three phases of this human investigation of the relative safety and tolerance of oral chlorine disinfectant ingestion. In several cases, statistically significant trends were associated with treatment; however, none of these trends were judged to have immediate physiological consequence. One cannot rule out the possibility that, over a longer treatment period, these trends might indeed achieve proportions of clinical importance. However, within the limits of the study, the relative safety of oral ingestion of chlorine dioxide and its metabolites, chlorite and chlorate, was demonstrated by the absence of detrimental physiological response.

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Environmental Health Perspectives
Vol. 46, pp. 63-71, 1982

Reaction Products of Aquatic Humic Substances with Chlorine

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A major concern of the chlorination of aquatic humic materials is the ubiquitous production of trihalomethanes. A large number of other chlorinated organic compounds, however, have been shown to be formed by chlorine's reaction with humic substances. In this study, humic material was concentrated from a coastal North Carolina lake and chlorinated at a chlorine to carbon mole ratio of 1.5 at pH 12. A high pH was necessary for complete dissolution of the humic material and for production of adequate quantities of oxidation and chlorination products for extraction, separation and mass spectrometric identification. After concentration in ether, samples were methylated, separated with a 50-m OV-17 glass capillary column or a 25 m SP-2100 fused-silica column and identified. A Hewlett-Packard 5710A gas chromatograph interfaced to a VG Micromass 7070F double-focusing mass spectrometer was used. Low resolution, accurate mass measurements were made with a combined EI-CI source.

The ability to do low resolution, accurate mass measurements made possible a rapid scan function necessary for capillary column gas chromatography. Accurate mass measurements allowed increased confidence in the identification of compounds, most of which are not available as standards. The products identified in these studies were chlorinated aliphatic straight-chain acids dominated by di- and trichloroacetic acid and the chlorinated dicarboxylic acids: succinic, fumaric and maleic acids. Chlorinated and unchlorinated aliphatic mono- and dicarboxylic acids and unchlorinated polycarboxylic aromatic acids comprise the remaining bulk of the compounds identified.

Introduction

Since Rook (*1*) first suggested that natural aquatic humic substances were responsible for the formation of the trihalomethanes in Rotterdam drinking water, a large number of studies have concentrated on the identification and quantification of these volatile, nonpolar dissolved compounds. Although natural organic materials in water (humic acid, fulvic acid, plant extractives, etc.) are procedurally defined and of unknown and presumable diverse structural composition, a body of data exists which supports the hypothesis that they are responsible for a large fraction of the chlorinated products produced in the chlorination of drinking water.

In comparing the total amount of organic halogen (TOX) produced in drinking water treatment to the level of trihalomethanes (THM), Oliver (*2*) found slightly larger TOX values than THM values. Glaze et al. (*3*) found five to six times higher amounts of TOX formation potential than THM formation potential using several procedures for this comparison. These findings suggest the need for increased identification of the nonvolatile chlorination products of the OCl^-/aquatic humic reaction. Such identification was the principal objective of the work reported here and earlier by the authors (*4*) and others (*5*).

The complexity of the OCl^-/aquatic humics reaction product mixture has made it necessary to refine the separation and mass spectral identification techniques reported earlier. The refinements have resulted in a considerable increase in the range and accuracy of product identification.

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Experimental

Humic Isolation and Chlorination

The isolation of humic material from the natural water system and its reaction with chlorine have been described previously (4). Briefly, humic acid was extracted from a highly colored surface water (Black Lake, Elizabethtown, N.C.) by acid precipitation at pH 2.2 (HCl), settling and centrifugation. After a cleanup procedure which included three rounds of redissolving in $0.5N$ NaOH, precipitation and centrifugation, the material was washed twice with pH 2.2 HCl solution and freeze-dried to a solid. For reaction with chlorine the solid material was dissolved in pH 12 NaOH solution, centrifuged and filtered for a final concentration of 420 mg/l. total organic carbon. This solution was then reacted for 48 hr at pH 12 with aqueous hypochlorite at a mole ratio (OCl^-/C) of 1.5. It is reasonable to assume that chlorination products produced at high pH do not differ qualitatively from those produced at more acidic pH values. However, greater yields of substitution products may occur at lower pH due to the increased concentration of HOCl. After this period excess chlorine was removed with sodium arsenite and the pH lowered to 1.0 with HCl. The solution was then extracted with an equal volume of redistilled diethyl ether which was then dried with sodium sulfate, concentrated in a Kuderna-Danish apparatus and methylated with diazomethane. Solvent blanks using redistilled solvent yielded no products.

GC/MS Procedures

The GC/MS facility used in these studies consists of a Hewlett-Packard 5710A capillary column gas chromatograph, interfaced to a VG Micromass 7070F double-focusing mass spectrometer equipped with a combined EI-CI source. Data reduction was performed by a VG Datasystems 2035 F/B computer and storage on Systems Industries compatible 6.6 megaword dual-density cartridge disks. A Versatec 800A electrostatic printer/plotter was used to output the data.

The gas chromatograph was fitted with a Grob-type split/splitless injector, supplied by GC^2 (chromatography) Ltd. P. O. Box 26, Northwich, Cheshire, UK. The nonpolar column used for the separations was a Hewlett-Packard 25 m SP-2100 fused-silica capillary, which was easily inserted within the 0.7 mm ID glass-lined tubing direct interface to the MS ion source as far as the quartz inlet restriction, thus eliminating most of the potential interface problems such as dead-volume or active sites. At a head pressure of 4 lb/in^2, the helium flow rate through this column was about 1 ml/min at 60°C. Analyses were also performed on a 50 m OV-17 Quadrex glass capillary column supplied by Applied Sciences, Inc. The flow rate through this column was about 0.8 ml/min at a head pressure of 10 lb/in^2. The program rate used on either column was usually 40°C/min after a 2-min delay from the injection temperature of 60°C, to a final temperature of 260°C.

The 7070F spectrometer is equipped with a Hall-probe regulated field-control system, enabling scan cycle times of around 1.2 sec. For low resolution accurate mass measurement, the cycle time was 3.0 sec over the mass range 450-20-450 (1.5 sec/decade, 1 sec interscan delay).

The standard operating conditions in EI mode were: accelerating voltage 4 kV, trap current 100 μA, electron energy 70 eV, source pressure 2×10^{-6} torr (1 ml/min helium flow), source temperature 200°C, resolution 1500. In CI mode, the reactant gas used was isobutane and the operating conditions were: filament current 500 μA, electron energy 100 eV, source pressure approximately 0.3 torr, source temperature 100°C, resolution 1000. For accurate mass measurements, a low resolution technique employing tetraiodoethylene as internal reference compound was used, enabling operation at full sensitivity.

Sample injection volumes were typically 1.5 μl, and split ratios of 5:1 to 10:1 were employed.

Bases of Structural Assignments from Mass Spectra

In general, the structure of an individual component was deduced by interpretation of the CI mass spectrum, which provided the molecular weight, and the EI accurate mass spectrum, which enabled assignment of elemental composition to the major peaks and hence the molecular formula in addition to providing a characteristic fragmentation pattern. In many cases, the identity of a component thus evaluated was confirmed by matching EI and CI mass spectra with those of an authentic standard specimen.

For example, scan #629 in the OV-17 chromatogram gave the EI spectrum shown in Figure 1. The accompanying CI spectrum indicates a compound of molecular weight 194, with the ion 31 mass units lower being indicative of a carboxylic acid methyl ester. The accurate mass of the molecular ion was determined to be 194.0568. Table 1 lists the five possible elemental formula choices for this mass within ± 10 ppm given the indicated maximum numbers of atoms for each element. Of these, only two are possible molecular ions. The others are

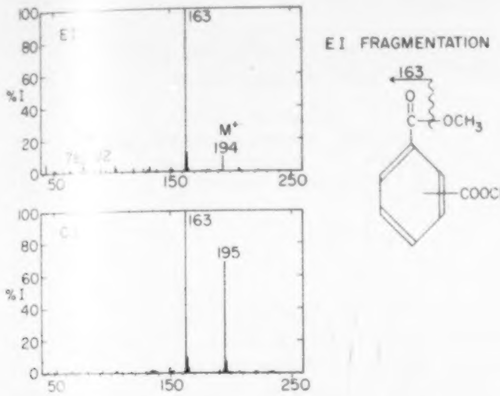

FIGURE 1. Mass spectra of a typical aromatic ester.

eliminated either because the mass spectrum shows that there is no chlorine in the molecule or the rules of valency are not satisfied. Thus, the choice is between only two possible formulae as compared for example with thirteen possible choices when the tolerance limit is increased to 100 ppm. Even this relatively low precision is already well beyond the scope of accuracy achievable using a quadrupole spectrometer. The utility of the technique of low resolution accurate mass measurement at scan speeds compatible with capillary column GC/MS is therefore intuitively obvious. The main prerequisites for such a system are a double-focusing mass spectrometer with a highly reproducible fast cycle scan function and appropriate computer programming.

Figures 1 and 2 are examples of aromatic and aliphatic diesters which were confirmed with standard compounds. Figure 2 shows a more typical situation; no molecular ion is observed in the EI spectrum, showing the utility of the CI spectrum which usually gave an M + H ion. Having both accurate mass data and mass fragmentation patterns on compounds of similar type gives a great deal of confidence to interpretations such as that shown in Figure 3 where no standard compound or library spectrum is available.

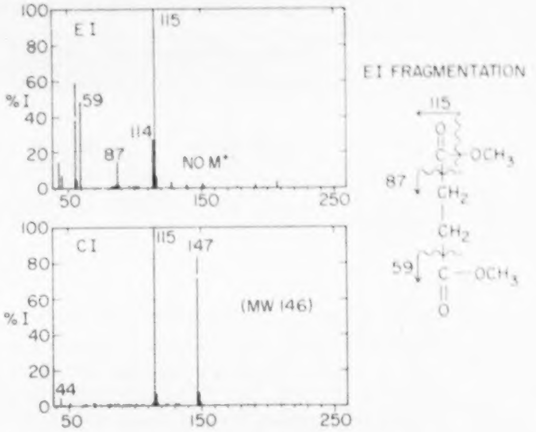

FIGURE 2. Mass spectra of a typical aliphatic ester.

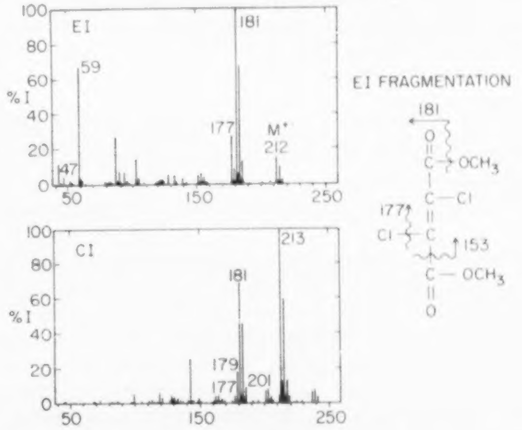

FIGURE 3. Mass spectra of a typical chlorinated ester.

Results and Discussion

Figure 4 shows the reconstructed gas chromatogram (RGC) of the methylated, ether-extractable, pH 12 chlorination products of aquatic humic acid analyzed with an SP-2100 fused-silica capillary col-

Table 1. Possible formulae within 10 ppm (2 mmu) for observed accurate mass 194.0568 ($M^{+\cdot}$).[a]

C	H	O	Cl	N	ppm
10[b]	10	4	0	0	–5.8
7	13	3	1	1	–8.3
8	8	3	0	3	1.1
5	11	2	1	4	–1.4
6[b]	6	2	0	6	8.0

[a] Given: $^{12}C/40$, $^{16}O/9$, $^{35}Cl/6$, $^{14}N/6$.

[b] Chemically feasible choices; possible formulae within 100 ppm total 37, of which 13 are chemically feasible.

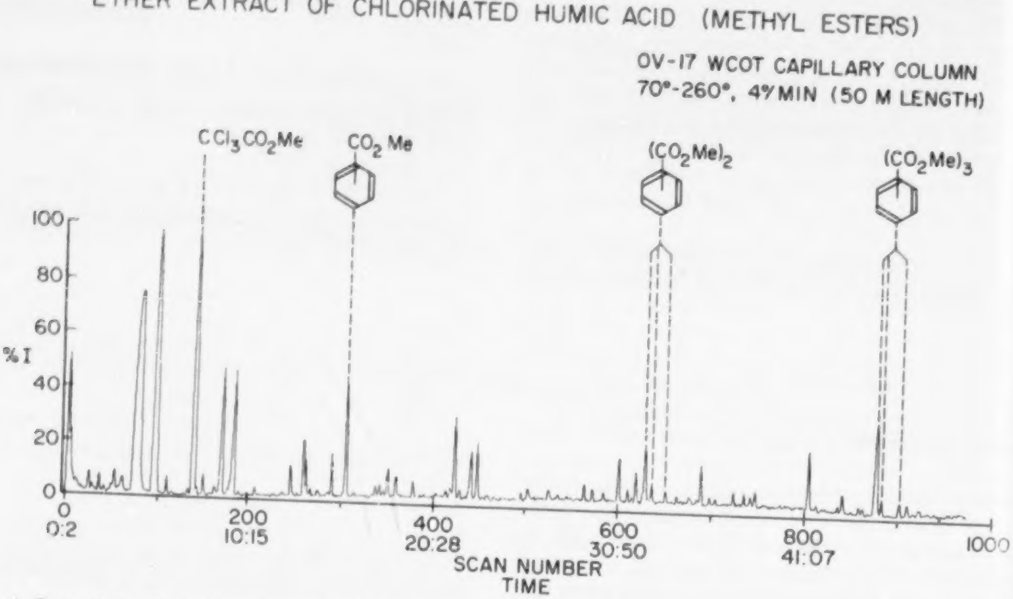

FIGURE 4. Reconstructed gas chromatogram of the methylated, ether-extractable, pH 12 chlorination products of aquatic humic acid analyzed with an SP-2100 fused-silica capillary column.

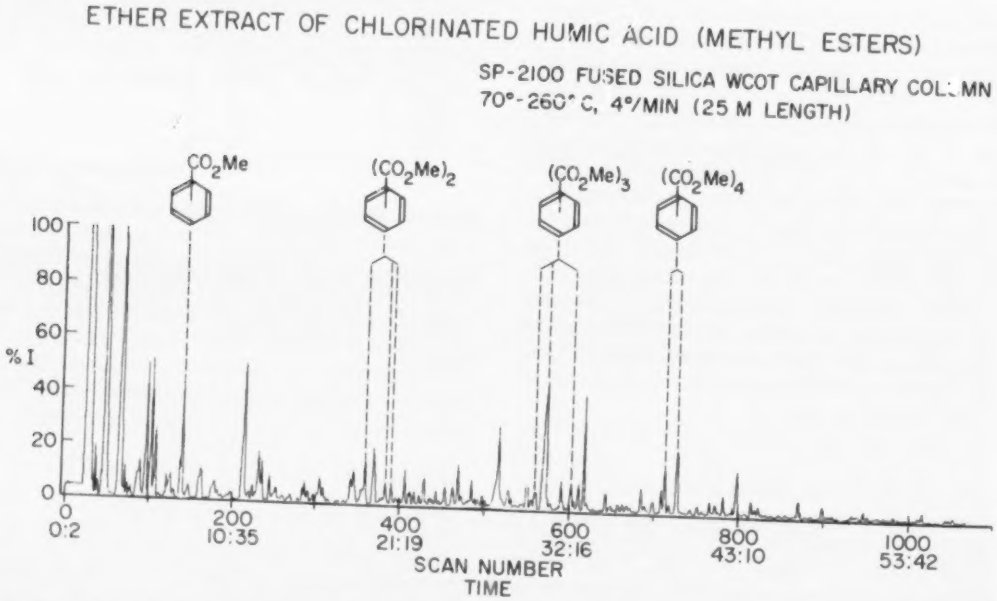

FIGURE 5. Reconstructed gas chromatogram of the methylated, ether-extractable, pH 12 chlorination products of aquatic humic acid analyzed with an OV-17 glass capillary column.

Table 2. Summary of components detected by GC/MS.

Type of compound	Number of compounds and method[a]	
	Packed	Capillary
Aliphatic monoesters	5 (1)	21 (1+2)
Aliphatic diesters	5 (1)	11 (1+2)
Aromatic esters	15 (1)	24 (1+2)
Chlorinated compounds	15 (1)	15 (1), 36 (2)
Others	0 (1)	38 (2)

[a](1) = OV-17; (2) = SP-2100.

umn. In the original packed column results (4), approximately 8% of the starting carbon appeared as chromatographable products. With the technique used it is not possible to analyze extremely low molecular weight materials such as haloforms or the high molecular weight, neutral or basic fractions, or other components nonextractable into ether from acid solution or nonchromatographable. In addition to the experimental advantages of the fused-silica capillary column, the 25 m column using the SP-2100 coating (Fig. 4) gave a wider boiling range of identified products than a 50 m glass capillary OV-17 column (Fig. 5). The latter column, however, gave better separations and both capillary columns produced much better results than those reported in the previous study (4) with the use of OV-17 with a packed column. For instance, the first of the dicarboxybenzoic acids in scan #359 in Figure 4 was not resolved from the next higher molecular weight material in the original packed column data. This dicarboxybenzoic acid, even with the SP-2100 fused-silica column, has a small unresolved shoulder. The extremely high performance of the OV-17 glass capillary column is clearly shown, as the first dicarboxybenzoic acid is completely resolved as a single component (scan #629 in Fig. 5).

Table 2 gives a summary of the number of components detected by each of the three chromatographic systems. The SP-2100 column with its wider boiling range separation eluted a larger number of compounds than the OV-17 column. The higher molecular weight materials above scan #600 only seen with the SP-2100 column were, however, in rather small yield.

Nearly all of the products identified in this experiment are esters presumably derived from the methylation of mono- and polybasic acids. These include mono- and dibasic, saturated and unsaturated, chlorine substituted and unsubstituted acids. In especially high yield are di- and trichloroacetic acid, dichlorosuccinic acid, and a series of isomers including dichloromaleic acid. A large number of mono- and dibasic unchlorinated aliphatic acids from acetic and oxalic acid up to the C_{27} monobasic fatty acid were identified. The dibasic unchlorinated aliphatic acids were generally low molecular weight containing from 2 to 10 carbons. The aliphatic acids may be ring-cleavage products. Most are also in relatively low yield indicated by one or two stars in the "Relative abundance" column shown in Tables 3 and 4. Aromatic acids including mono- to hexacarboxybenzoic acid in all isomers as well as small quantities of methyl-substituted aromatic acids and isomers of carboxyl-substituted α-ketobenzoic acid were also detected. Noticeably missing from the aromatic series are chlorine-substituted aromatic acids and aromatic acids with aliphatic side chains other than methyl. This pattern is similar to that found with permanganate oxidation, suggesting that chlorine in alkaline solution is capable of oxidizing side chains down to terminal carboxyl groups on the aromatic ring.

The products found in this study are similar to those previously identified (4), but as shown in Table 2, a significant number of additional compounds are reported here. In addition, the separation of multicomponent peaks made possible by capillary gas chromatography has also improved the accuracy of the mass measurements made, providing more confidence in the elemental formulae assigned and structures determined.

Summary and Conclusions

Tables 3 and 4 list the assignments made for the chromatograms shown in Figures 4 and 5. Along with the proposed structure or elemental formula, a scan number is listed for reference back to Figures 4 and 5 as well as the relative abundance. The highest or most abundant yield of product is shown as five stars down to the lowest or least abundant yield as a single starred component. Also listed are comments as to confidence in assigned structure and other comments. The phrase "standard confirmed" notes those compounds in which authentic standards have been used to confirm structures.

The relatively high yields of di- and trichloroacetic acid, although they are quite low in molecular weight, extremely acidic and polar, making their extraction into ether relatively inefficient, would suggest that

Table 3. Chlorination products identified on SP-2100.

Proposed structure or formula	Scan number	Relative abundance	Comments
C_2HOCl_3	34	***	Possible solvent impurity
$ClCH_2—COOCH_3$	37	***	Confident
Aliphatic	39	**	Possible solvent impurity
$Cl_2CH—COOCH_3$	46	*****	Standard confirmed
Unknown	51	***	Possible solvent impurity
$Cl_3C—COOCH_3$	64	*****	Standard confirmed
$Cl_2C=CH—COOCH_3$ (isomer)	72	**	Tentative
$C_3H_5O_2Cl$	74	*	Tentative
Unknown	75	**	
$Cl_2C=CH—COOCH_3$ (isomer)	77	*	Isomer of 72, tentative
Ar—COH	79	*	Confident
Dichlorinated ester	85	*	Tentative
$Cl_3C—COH$	88	*	Tentative
$C_5H_7O_2N$	90	***	Tentative
Unknown aliphatic compound	95	**	
Unknown aliphatic compound	97	***	
Unknown aliphatic compound	101	**	
Unknown aliphatic compound	104	*****	
$H_3COOC—(CH_2)_2—COOCH_3$	108	***	Standard confirmed
$C_3H_4O_2Cl_2$	122	*	Tentative
Aliphatic diester	125	*	Tentative
Monochlorinated aliphatic diester	127	*	Tentative
Unknown	130	*	
Ar—$COOCH_3$	138	***	Standard confirmed
Chlorinated aliphatic ester	145	*	Tentative
Dichlorinated aliphatic compound	148	*	Tentative
$H_3COOC—CH=CCl—COOCH_3$ (isomer)	153	*	Tentative
$H_3COOC—CH_2—CHCl—COOCH_3$	160	*	Confident
$H_3COOC—CH_2—CHCl—COOCH_3$	163	**	Isomer of 160, tentative
Aliphatic diester	176	*	Tentative
Aliphatic diester	180	***	Tentative
Aliphatic ester	183	*	Tentative
Unknown	197	*	
Dichlorinated aliphatic ester	213	***	Tentative
$H_3COOC—C_2H_2Cl_2—COOCH_3$ (isomer)	215	*****	Tentative
$H_3C—(CH_2)_7—COOCH_3$	221	*	Confident
Trichlorinated aliphatic ester	224	*	Tentative
$H_3COOC—CCl=CCl—COOCH_3$	230	*	Isomers, *cis* and *trans*
	232	**	
Tetrachlorinated aliphatic ester	245	*	Tentative
Unknown	247	*	
Unknown	252	*	
Aliphatic diester	260	*	Tentative
Aliphatic diester	270	*	Tentative
$H_3C—(CH_2)_8—COOCH_3$	284	*	Tentative
Ar—$CHCl—COOCH_3$	287	**	Tentative
$H_3COOC—(CH_2)_5—COOCH_3$	291	*	Confident
$C_7H_8O_4Cl_2$ aliphatic ester	297	*	Tentative
Dichlorinated aliphatic ester	305	*	Tentative
Dichlorinated aliphatic aldehyde or ketone	309	**	Tentative
$C_8H_{10}N_2O_4$	342	**	Very tentative, note homologs at 510, 513, 516
	345		
$H_3COOC—(CH_2)_6—COOCH_3$	354	*	Confident
Ar—$(COOCH_3)_2$	359	****	Standard confirmed
Trichlorinated aliphatic ester	369	**	Tentative
Chlorinated ester	371	*	Tentative
Aliphatic ester	377	*	Tentative
Ar—$(COOCH_3)_2$ + chlorinated compound	383	*	Standard confirmed
Ar—$(COOCH_3)_2$	389	*	Standard confirmed
H_3C—Ar—$(COOCH_3)_2$	404	*	Tentative
$H_3C—(CH_2)_{10}—COOCH_3$	406	**	Confident
Aliphatic diester	410	*	Tentative
Chlorinated aliphatic diester	413	*	Tentative
$C_8H_8O_5$	417	*	Tentative
Aliphatic ester	428	*	Tentative
Ester	429	*	Tentative

Table 3. *(Continued)*

Proposed structure or formula	Scan number	Relative abundance	Comments
$C_6H_{11}O_3Cl$ ester	439	*	Tentative
$H_3C—(CH_2)_{11}—COOCH_3$	443	*	Confident
$C_8H_8O_5$	454	**	Homolog of 417, tentative
Chorinated ester	462	*	Tentative
$H_3C—(CH_2)_{11}—COOCH_3$ + chlorinated ester	463	*	Confident
$C_9H_7O_2Cl$ ester + $C_8H_9O_6Cl$ ester	469	**	Tentative
Isomer of 345	474	*	Tentative
$C_6H_8O_3Cl$ ester	482	*	Tentative
Aliphatic ester	485	*	Tentative
Aliphatic monoester	489	*	Tentative
Unknown mixture	492	*	
$H_3C—(CH_2)_{12}—COOCH_3$	497	*	Confident
$C_7H_8N_2O_4$	510	*	Homologs of 342, 345, 474, tentative
	513	**	
	516	***	
$H_3C—(CH_2)_{12}—COOCH_3$	517	***	Confident
$C_{12}H_{13}O_4Cl_3$	523	*	Tentative
Complex aliphatic ester	528	*	Tentative
$C_{11}H_{12}O_3Cl_4$ ester	523	*	Tentative
Aliphatic monoester	543	*	Tentative
$H_3C—(CH_2)_{13}—COOCH_3$	550	**	Confident
$Ar—(COOCH_3)_3$ + another aromatic	558	*	Standard confirmed
$Ar—(COOCH_3)_3$	566	***	Standard confirmed
$C_{10}H_9O_4Cl_3$ ester	577	*	Tentative
Complex aliphatic ester	589	**	Tentative
$Ar—(COOCH_3)_3$	601	**	Standard confirmed
$C_8H_9O_5Cl_3$ ester	604	*	Tentative
$H_3C—Ar—(COOCH_3)_3$ + complex chlorinated ester	610	*	Tentative
$H_3C—(CH_2)_{14}—COOCH_3$	616	****	Confident
Complex aliphatic compound	619	*	Tentative
$H_3COOCOC—Ar—(COOCH_3)_2$	641	**	Very tentative
$C_{11}H_9O_5Cl_3$ ester	647	*	Tentative
$C_{12}H_8O_5Cl_2$ ester	651	*	Tentative
$C_{12}H_{10}O_2Cl_4$ ester	657	*	Tentative
Mixture of esters	662	*	Tentative
$H_3C—Ar—(COOCH_3)_3$ + another compound(s)	666	*	Tentative
Complex aliphatic compound	684	**	Tentative
$H_3COOCOC—Ar—(COOCH_3)_2$ + $H_3C—Ar—(COOCH_3)_3$	688	*	Very tentative
$Ar—(COOCH_3)_4$	697	*	Standard confirmed
$H_3C—(CH_2)_{16}—COOCH_3$	708	*	Confident
$Ar—(COOCH_3)_4$	712	***	Standard confirmed
Aliphatic diester	716	*	Tentative
$Ar—(COOCH_3)_4$	726	****	Standard confirmed
$(H_3C)_2—Ar—(COOCH_3)_3$	750	*	Tentative
$H_3COOCOC—Ar—(COOCH_3)_3$	764	*	Very tentative
Complex aliphatic compound	771	*	Tentative
$H_3COOCOC—Ar—(COOCH_3)_3$	781	**	Very tentative
$H_3C—(CH_2)_{18}—COOCH_3$	792	*	Confident
$H_3COOCOC—Ar—(COOCH_3)_3$	797	***	Very tentative
$H_3COOCOC—Ar—(COOCH_3)_3$	814	*	Very tentative
$Ar—(COOCH_3)_5$	822	*	Standard confirmed
Phthalate	869	*	Confident
$H_3C—(CH_2)_{20}—COOCH_3$	871	*	Confident
$H_3COOCOC—Ar—(COOCH_3)_4$	883	*	Very tentative
$H_3COOCOC—Ar—(COOCH_3)_4$	897	*	Very tentative
$H_3C—(CH_2)_{21}—COOCH_3$	909	*	Confident
$C_{18}H_{16}O_8$ ester	932	*	Tentative
$H_3C—(CH_2)_{22}—COOCH_3$	946	*	Confident
$C_{18}H_{16}O_8$ ester	950	*	Tentative
$H_3C—(CH_2)_{23}—COOCH_3$	981	*	Confident
$H_3C—(CH_2)_{24}—COOCH_3$	1015	*	Confident
$C_{20}H_{18}O_{10}$ ester	1043	*	Tentative
$C_{20}H_{18}O_{10}$ ester + $H_3C—(CH_2)_{25}—COOCH_3$	1051	*	Tentative, confident

Table 4. Chlorination products identified on OV-17.

Proposed structure or formula	Scan number	Relative abundance	Comments
$ClCH_2—COOCH_3$	53	**	Confident
$H_3C—COOC_2H_5$	80	*****	Solvent
$Cl_2CH—COOCH_3$	96	*****	Standard confirmed
$H_3COOC—COOCH_3$	110	**	Confident
Unknown	134	*	Related to 143, 171, 183
$Cl_3C—COOCH_3$	137	*****	Standard confirmed
Unknown	143	*	Related to 134, 171, 183
$Cl_2C—CH—COOCH_3$ (isomer) + $H_3C—COOCH_2Cl$	150	**	Tentative
$H_3COOC—CH_2—COOCH_3$	162	*	Confident
Unknown	171	*****	Related to 134, 143, 183
$Cl_3C_2H_2—COOCH_3$	176	*	Very tentative
Unknown	183	*****	Related to 134, 143, 171
$Ar—COH$	207	*	Confident
$Cl_2C—C_3H_7—COOCH_3$	245	**	Tentative, isomers
$H_3COOC—(CH_2)_2—COOCH_3$	259	***	Standard confirmed
$C_5H_7NO_2$	262	**	Tentative
$H_3C(Cl)C—(COOCH_3)_2$ or $ClH_2C—CH—(COOCH_3)_2$	290	***	Tentative
$Ar—COOCH_3$	305	*****	Standard confirmed
$H_5C_2—CH—(COOCH_3)_2$	337	*	May be interchanged with 341, tentative
$H_3COOC—CH_2—CH(CH_3)—COOCH_3$	341	*	May be interchanged with 337, tentative
$H_3COOC—CH_2—CHCl—COOCH_3$ + another methyl ester	351	**	Confident
$H_3COOC—CCl_2—COOCH_3$	360	**	Confident
$H_3COOC—(CH_2)_3—COOCH_3$	378	**	Confident
$H_3COOC—CCl_2—CH_2—COOCH_3$	423	****	Tentative
$H_3COOC—(CH_2)_4—COOCH_3$	428	**	Confident
$H_3COOC—CCl=CCl—COOCH_3$	441	**	Isomers, *cis* and *trans*
	447	***	
$H_3COOC—(CH_2)_5—COOCH_3$	503	**	Confident
$Cl—C_7H_6—COOCH_3$	525	**	Isomers, tentative
	535	**	
$H_3C—(CH_2)_{10}—COOCH_3$	564	**	Confident
$H_3COOC—(CH_2)_6—COOCH_3$	572	**	Confident
$[C_3H_2Cl_2O]—(COOCH_3)_2$	584	**	Very tentative
$C_{13}H_{10}O_2$ or $C_8H_{10}N_2O_4$	601	***	Tentative
$Ar—(COOCH_3)_2$	629	****	Standard confirmed
$Ar—(COOCH_3)_2$ + $H_3COOC—(CH_2)_7—COOCH_3$	636	*	Standard confirmed
$Ar—(COOCH_3)_2$	651	**	Standard confirmed
$H_3C—(CH_2)_{12}—COOCH_3$	688	*	Confident
$H_3COOC—(CH_2)_8—COOCH_3$	698	*	Confident
$H_3C—Ar—(COOCH_3)_2$	704	*	Tentative
$H_3C—(CH_2)_{13}—COOCH_3$	724	*	Isomers, confident
	730	*	
	747	*	
$H_3C—(CH_2)_{14}—COOCH_3$	805	***	Confident
$C_{14}H_{12}O_2$	840	**	Possible artifact
$Ar—(COOCH_3)_3$	878	*****	Standard confirmed
$Ar—(COOCH_3)_3$	883	**	Standard confirmed
$Ar—(COOCH_3)_3$	902	*	Standard confirmed
$H_3C—(CH_2)_{16}—COOCH_3$	910	*	Confident

these compounds are major products of the chlorination of aquatic humic materials. These compounds, because of their concentration, are likely candidates for additional toxicology and mutagenicity testing. It is important to note that chlorinated aromatic structures which are commonly found in toxic materials are completely absent from the structures identified in this study.

Three major factors in the analytical procedure employed were crucial to the success of the compound identifications. These are: (1) the use of fused-silica capillary GC columns with their flexibility and high separation efficiency; (2) the ability to employ scan rates compatible with the sharp elution profiles of the GC peaks using a double-focusing sector mass spectrometer and; (3) the acquisition of accurate mass data without serious compromise to the sensitivity or scan speed. Item three results from the fact that low resolution was employed in these analyses. If high resolution had been used, sensitivity would have suffered, and scan rates would have had to be slower to obtain an undistorted spectrum. This is incompatible with the very sharp peaks eluting from capillary columns. Compared with the typical nominal mass data obtained with quadrupole spectrometers and the use of library search techniques, we are able to identify many more components with a much greater degree of certainty.

This research was supported in part by EPA research grant R-804430 from the Municipal Environmental Research Laboratory, Cincinnati, Ohio (Alan A. Stevens, Project Officer).

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Environmental Health Perspectives
Vol. 46, pp. 73-86, 1982

Implications of Treating Water Containing Polynuclear Aromatic Hydrocarbons with Chlorine: A Gas Chromatographic-Mass Spectrometric Study

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The products of aqueous chlorination reactions of 1-methylnaphthalene, fluorene, dibenzofuran, anthracene, phenanthrene, 1-methylphenanthrene, fluoranthene, and pyrene have been determined. The conditions employed for these reactions approximated those that might be encountered in water treatment facilities. Reactions at pH > 6 tended to produce oxygenated products (epoxides, phenols, quinones, etc.), and reactions at pH < 6 tended to produce both oxygenated (quinones) and chlorinated products.

The use of chlorination as the predominant technique for water renovation and disinfection has been questioned because of the reaction of active chlorine species with organic compounds present in the water to form products which may be biologically harmful (*1-3*). These reactions may occur at the site of chlorine addition as well as throughout a water distribution system (*4*). Polynuclear aromatic hydrocarbons (PAH) have been suggested as the precursors to at least a portion of the mutagens produced in some chlorination processes (*4*). The levels of these ubiquitous PAH compounds may be increased by the presence of coal tar coatings inside pipes and water storage tanks (*5*).

In order to provide further insight into the possible role of PAH in chlorine-induced mutagen formation, this laboratory has continued the study of the aqueous chlorination chemistry of PAH (*6*, *7*). The present report describes a detailed study of the product distributions of several PAH compounds which were chosen for study based on their previous identification in drinking water (*8*). A related study in these laboratories of the reaction kinetics and mechanisms for the aqueous chlorination of phenanthrene, fluorene, and fluoranthene will be reported elsewhere.

Experimental

Procedure

Twelve liters of water were treated with chlorine gas, and an appropriate amount of sodium hydroxide was added to achieve the desired pH. A solution of the PAH in acetonitrile (40-800 ml, specific conditions in Tables) was added to initiate the reaction which was monitored by HPLC. For high pH solutions, the pH was maintained by periodic addition of NaOH. To this, 2.5-3 equivalents (based on the total chlorine concentration) of dimethyl sulfoxide or sodium thiosulfate were added to terminate the reaction. In some cases, the reactant solution was acidified with sulfuric acid in order to suppress ionization of acidic products. The solution was then forced through two 7 × 50 mm stainless steel adsorption columns connected in series. The first column contained C-18 Porasil B (37-75 μm, Waters Asso-

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ciates), and the second contained XAD-2 (100 μm, Rohm and Haas). Reaction products which were adsorbed were later removed by elution of the column train with acetonitrile and then with methylene chloride. Some separation of these products was observed during the elution process. The various fractions collected were then checked for product content by HPLC and concentrated under a nitrogen stream prior to analysis by gas chromatography-mass spectrometry (GC/MS). Portions of fractions suspected of containing acidic compounds were methylated with diazomethane.

Standards

The following compounds were purchased, checked for purity, and, where necessary, purified by recrystallization, sublimation, and/or preparative reversed phase liquid chromatography: phenanthrenequinone, 9-chlorophenanthrene, 9-fluorenone, 1-chloropyrene.

The following compounds were prepared according to literature methods: *trans*-9,10-dihydro-9,10-dihydroxyphenanthrene (*9*), 2-chlorodibenzofuran (*10*), 2,8-dichlorodibenzofuran, (*10*), phenanthrene-9,10-oxide, (*11*), 2,3-fluoranthenedione (*12*), 3-fluoranthenol (*13*), 2-chlorofluorene (*14*), 3-chlorofluoranthene (*14*), 4-pyrenol (*15*), 9-fluorenone-1-carboxylic acid (*16*), 4,5-pyrenedione (*17*), *cis*-4,5-dihydro-4,5-dihydroxypyrene (*18*), dichloropyrene (*19*, *20*), 1,6-/1,8-pyrenedione (*21*), and 4,5-phenanthrenedicarboxylic acid anhydride (*22*).

9-Phenanthrenol was prepared by the rearrangement of phenanthrene-9,10-oxide with base. Diisopropylamine (0.476g, 0.0047 mmole) and *n*-butyllithium (3.23 ml of 1.6*M* solution in hexane, 0.0052 mole) were combined in tetrahydrofuran (THF) at –78°C under N_2. A slurry of phenathrene-9,10-oxide (0.911 g in THF, 0.0047 mole) was added by syringe and the mixture was then allowed to warm slowly overnight. Sulfuric acid (1*N*) was added, and the mixture was twice extracted with benzene and the combined extracts were dried with magnesium sulfate and evaporated in a rotary evaporator at 30°C. Tan crystals (0.844 g, 92%) resulted with mp 144–147°C. Recrystallization from benzene gave tan crystals, mp 149-151°C (lit. mp 151–152°C) (*23*). IR(KBr) showed the absence of phenanthrenequinone. This phenol undergoes rapid autoxidation to phenanthrenequinone (*24*), at such a rate as to preclude the preparation of pure solutions. The generation of an HPLC standard curve required correction for the amount of quinone present.

9,10-Dichlorophenanthrene was prepared by the chlorination of phenanthrene. Methylene chloride (100 ml) and commercial bleach (100 ml) were combined in a closed container and the pH of the aqueous layer adjusted to 4 with phosphoric acid (*11*). The total chlorine content of the two layers was determined by iodometric titration to be 0.03*M*. Phenanthrene (0.85 g, 0.0048 mole) was added and the mixture stirred at room temperature for 5 hr. The organic layer was then removed, washed with water and saturated with sodium thiosulfate solution, dried over magnesium sulfate and evaporated to yield a yellow solid (80%). Recrystallization from methanol and again from acetonitrile gave tan crystals of mp 157.5-159°C (lit. mp 158-160°C) (*19*); NMR ($CDC1_3$) δ 7.6-8.1 (*m*, 4H), 8.4-9.0 (*m*, 4H).

2,7-Dichlorofluorene was prepared from the bis-diazonium salt. 2,7-Diaminofluorene (1.0 g, 1 mmole, Aldrich Chemical Co.) was placed into 4 ml of water and added to 11 ml of 4.4*N* hydrochloric acid. Sodium nitrate (0.155 g, 2.2 mmole) in 4 ml of water was then added and the resulting mixture was stirred for 1.5 hr at 20°C. This mixture was then added to a slurry of cuprous chloride (0.385 g, 4.3 mmole) in 8 ml of water. After 4 hr at 50°C the resulting solid was removed, taken up into methylene chloride and washed with 1*N* hydrochloric acid, 1*N* sodium hydroxide, and water. The organic layer was then dried over magnesium sulfate and evaporated. Recrystallization of the solid from hexane and then from methanol gave 2,7-dichlorofluorene, a white solid of mp 126°C (lit. mp 128°C) (*25*); NMR ($CDC1_3$) δ 3.9 (*s*, 2H), 7.2-7.9 (*m*, 6H).

4,5-Phenanthrenedicarboxylic acid was prepared from the corresponding pyrenedione by a procedure similar to that reported for oxidation of fluoranthenequinone (*16*). 4,5-Pyrenedione (0.045 g, 0.195 mmole) was dissolved in 25 ml of tetrahydrofuran, and 10 ml of 30% H_2O_2 was added. After stirring overnight, the solution was acidified to pH 2 with hydrochloric acid and the product was extracted with methylene chloride, dried, and evaporated. Crystals (0.018, 35% yield) formed and were recrystallized from methanol: mp = 256°C (lit. mp = 258°C) (*22*). Mass spectrum of product after diazomethane treatment: *m/e* = 294(10), 235(100), 2[illegible]0(46).

Instruments and Apparatus

The gas chromatograph-mass spectrometer (GC-MS) was a Hewlett-Packard 5993B quadrupole, EI-70 eV equipped with an Avondale B capillary inlet and a 21 MX-E computer (ANSWER software). The GC/MS interface was modified to allow the entire effluent from a fused silica capillary columns to be drawn into the mass spectrometer. The column was either a 25 m (0.32 mm ID) SE-54 (siloxane, Hewlett-Packard) or a 15m (0.32 ID) polymethyl (5% phenyl) siloxane (J & W Scientific). Splitless injections were employed with 5-8 psi head pressure and 2 ml/min He carrier flow. A typical temperature program was 70 to 310°C at

10°C/min. The mass range scanned was 50 to 400 amu with a scan time of 1.6 sec.

The reversed-phase high performance liquid chromatography (HPLC) apparatus consisted of a Perkin-Elmer Series 3 microprocessor-controlled gradient system equipped with a Rheodyne 7105 injector. The analytical column was a Perkin-Elmer C-18 reversed-phase column, 0.26 × 25 cm (P.N. 089-0716). The column was surrounded by a glass jacket and the temperature was maintained at 27°C with a refrigerated/heated bath and circulator to insure reproducibility. A Waters Associates Model 440 fixed wavelength detector and a Perkin-Elmer LC-75 variable wavelength ultraviolet detector were connected in series. Data was acquired on a Waters Associates Data Module. In order to improve the useful sensitivity of this system in an in-line trap column was used. The trap column consisted of two 60 × 0.7 cm stainless steel columns packed with 37-75 μm C-18 Porasil B (Waters Assoc.) and was located between the water pump and the solvent-mixing coil. Acetonitrile was of HPLC grade (Fisher Scientific) and the water was obtained from a Millipore Corporation purification system (Milli-Q) fitted with an additional Continental Model 2021 canister.

Results and Discussion

Equilibria Considerations

The predominant chlorine-containing species in aqueous chlorine solutions are HOCl, OCl^-, Cl^-, Cl_2, and Cl_3^- (*26*). The relationships between the species are summarized by Eqs. (1), (2) and (3) (*27–29*). The OCl^- and HOCl concentrations are significant at pH greater than ~ 6 and the HOCl, Cl_2 and Cl_3^- concentrations are significant at pH less than ~ 6.

$$HOCl \rightleftharpoons H^+ = OCl^- \tag{1}$$

with

$$K_{HOCl} = 2.62 \times 10^{-8} \text{ at } 20°C$$

$$Cl_2 + H_2O \rightleftharpoons HOCl + H^+ + Cl^- \tag{2}$$

with

$$K_{Cl_2^-} = 3.38 \times 10^{-4} \text{ at } 20°C$$

$$Cl_2 + Cl^- \rightleftharpoons Cl_3^- \tag{3}$$

with

$$K_{Cl_3^-} = 0.19 \text{ at } 25°C$$

Table 1. Products of 1-methylnaphthalene reactions.

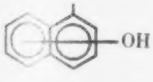
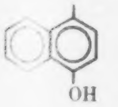
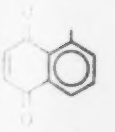
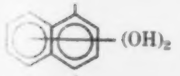
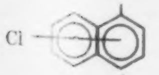
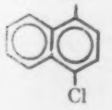
Proposed structure	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
$C_9H_8O_2$	1.051	148	—	Minor	148 (6), 133 (15), 120 (4), 105 (100), 91 (18), 77 (89)
$C_9H_8O_2$	1.106	148	—	Trace	148 (7), 133 (71), 105 (100), 77 (55)
	1.458	158	Minor	—	158 (100), 143 (2), 141 (4), 130 (16), 129 (38), 128 (29), 127 (16), 115 (15), 103 (8), 102 (10)
	1.556	158	Minor	—	158 (100), 157 (70), 143 (1), 141 (3), 130 (13), 129 (30), 128 (39), 127 (18), 115 (15), 103 (6), 102 (5)
	1.355	172	Minor	—	172 (100), 157 (5), 144 (19), 127 (16), 117 (8), 116 (54), 115 (77), 90 (46), 89 (54), 63 (48)
	1.442	174	Minor	Trace	174 (10), 159 (100), 131 (39), 105 (12), 103 (28), 77 (33)
	1.358	176	Minor	—	176 (64), 178 (26), 141 (100), 138 (37), 115 (40)
	1.375[d]	176	Minor	Major	176 (65), 178 (24), 142 (12), 141 (100), 139 (36), 115 (34)

Table 1. *(continued)*

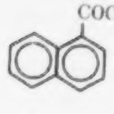
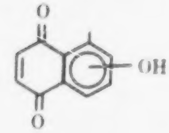
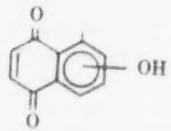
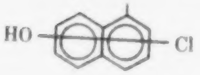
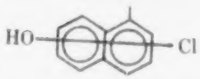
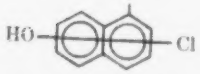
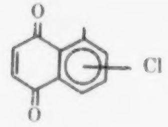
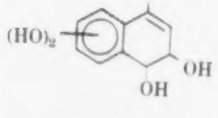
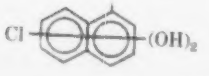
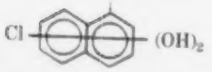
Proposed structure	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
Unknown	1.587	?	—	Minor	181 (92), 183 (32), 147 (100), 91 (34), 90 (43), 89 (55), 70 (49)
	1.541[c]	186	—	Trace	186 (47), 155 (100), 127 (60)
	1.414	188	—	Minor	188 (72), 173 (100), 161 (28), 160 (87), 132 (19), 131 (43), 105 (34), 104 (21), 103 (31), 89 (79), 77 (51)
	1.511	188	—	Minor	188 (90), 160 (13), 132 (9), 131 (8), 119 (76), 104 (22), 103 (100), 91 (70), 90 (70), 89 (71), 77 (59)
	1.599	192	Minor	—	192 (100), 194 (37), 177 (1), 157 (85), 129 (38), 128 (75), 127 (61), 126 (21), 102 (24), 77 (36), 64 (50), 63 (54)
	1.880	192	Minor	—	192 (100), 194 (37), 177 (2), 163 (20), 157 (46), 139 (17), 129 (53), 128 (22), 127 (55), 126 (18), 102 (17), 101 (15), 77 (29), 76 (21), 75 (27), 74 (23), 63 (50)
	1.897	192	Minor	—	192 (100), 194 (37), 177 (2), 165 (9), 163 (22), 157 (42), 129 (38), 128 (55), 127 (45), 126 (15)
Unknown	1.582	?	—	Minor	195 (100), 197 (39), 180 (8), 178 (7), 160 (6), 143 (4), 131 (24), 115 (8), 103 (37), 89 (7), 77 (24)
	1.728	206	Minor	—	206 (71), 208 (25), 180 (8), 179 (8), 178 (17), 163 (8), 165 (2), 150 (20), 1[illegible] (17), 143 (22), 126 (8), 124 (23), 115 (100), 114 (18), 89 (49), 87 (22), 86 (22), 75 (17), 74 (18), 73 (14), 63 (52)
	1.496	208	—	Minor	208 (5), 190 (9), 175 (26), 167 (34), 165 (100), 147 (15), 145 (12), 129 (12), [illegible]5 (18)
	1.481	208	Major	—	208 (10), 210 (4), 182 (16), 180 (4[illegible]), 167 (16), 165 (41), 145 (100), 144 (23), 116 (17), 115 (40), 102 (41), 101 (43), 77 (25), 75 (50)
	1.772	208	Minor	—	208 (8), 210 (4), 195 (30), 193 (76), 173 (100), 167 (8), 165 (19), 157 (24), 155 (11), 129 (15), 128 (10), 127 (17), 115 (15), 105 (50), 102 (35), 101 (36)

Table 1. *(continued)*

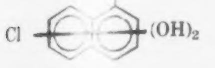
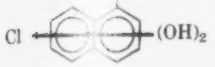
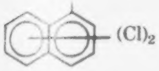
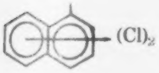
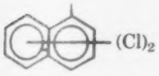
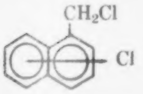
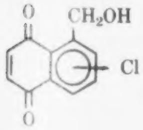
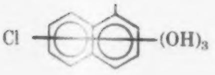
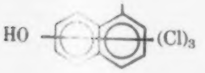
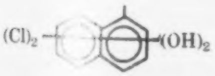
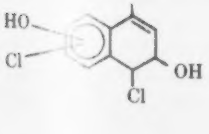
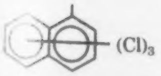
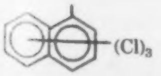
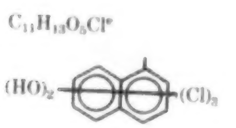
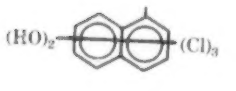
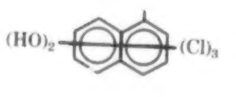
Proposed structure	Relative retention time (GC)^a	Molecular ion, m/z	Amt present		m/z (relative abundance)
			pH 3^b	pH 8^c	
	1.784	208	Minor	Minor	208 (11), 210 (4), 195 (29), 193 (80), 173 (100), 167 (8), 165 (22), 157 (24), 155 (11), 129 (15), 128 (12), 127 (18), 115 (20), 105 (44), 102 (32), 101 (29)
	1.797	208	Minor	—	208 (72), 210 (20), 182 (6), 180 (14), 167 (19), 165 (25), 155 (17), 154 (27), 153 (36), 152 (88), 145 (34), 124 (71), 117 (44), 115 (11), 103 (18), 89 (87) 62 (100)
	1.674	210	Major	Trace	210 (57), 212 (34), 177 (35), 175 (100), 149 (14), 139 (70), 113 (8)
	1.686	210	Major	Trace	210 (60), 212 (35), 177 (33), 175 (100), 151 (4), 149 (14), 139 (56), 113 (6)
	1.712	210	Minor	Trace	210 (43), 212 (24), 177 (33), 175 (100), 139 (38)
	1.804	210	Minor	—	210 (60), 212 (33), 177 (37), 175 (100), 149 (16), 139 (52)
	1.812	222	—	Trace	222 (63), 224 (23), 223 (16), 221 (28), 207 (8), 205 (10), 187 (23)
	1.694	224	—	Major	224 (3.8), 226 (1.4), 209 (9), 189 (18), 183 (5), 181 (16), 173 (13), 171 (23), 161 (100), 149 (11), 147 (24), 143 (35), 91 (39), 89 (52), 77 (74)
	1.916	226	Minor	—	216 (100), 228 (56), 211 (4), 209 (5), 193 (29), 192 (34), 191 (72), 173 (11), 165 (17), 164 (25), 163 (45), 162 (52), 137 (4), 128 (51), 127 (85), 126 (58), 113 (8)
	1.855	242	—	Minor	242 (15), 244 (8), 209 (4), 207 (16), 181 (36), 179 (92), 171 (37), 143 (18), 116 (38), 115 (100), 89 (38)
	1.820	244	Minor	—	244 (4), 246 (3), 211 (22), 210 (14), 209 (54), 208 (16), 193 (25), 192 (17), 191 (30), 180 (15), 179 (23), 173 (100), 165 (30), 163 (39), 162 (23), 157 (13), 145 (22), 142 (42), 129 (20), 128 (44), 127 (42), 126 (18), 117 (17), 116 (18), 115 (62)
	1.945	244	Minor	—	244 (50), 246 (49), 211 (68), 210 (20), 209 (100), 185 (9), 183 (13), 175 (29), 173 (67), 137 (22)
	1.961	244	Minor	—	244 (55), 246 (56), 211 (70), 209 (100), 185 (11), 183 (18), 175 (28), 173 (69), 137 (25)

Table 1. *(continued)*

Proposed structure	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
$C_{13}H_{15}O_3Cl$[e] or $C_{12}H_{11}O_4Cl$[e]	2.294	254	Minor	—	254 (26), 256 (10), 241 (34), 239 [illegible]00), 218 (10), 211 (9), 209 (19), 208 (17), [illegible]07 (65), 206 (32), 203 (7), 195 (13), 193 [illegible]1), 175 (15), 171 (26), 163 (16), 159 (37), [illegible]47 (65), 143 (21), 135 (15), 115 (42), 10[illegible]46)
$C_{11}H_{13}O_5Cl$[e]	2.370	260	Minor	—	260 (100), 262 (36), 261 (55), 259 [illegible]81), 246 (0.4), 244 (3), 233 (4), 231 (10)
$C_{11}H_{13}O_5Cl$[e]	2.441	260	Minor	—	260 (100), 262 (36), 261 (49), 259 [illegible]6), 233 (6), 231 (10)
	1.968	276	—	Trace	276 (48), 278 (53), 243 (4), 241 (10), 226 (3), 215 (51), 214 (20), 213 (100), 212 (20), 207 (9), 205 (52), 187 (19), 185 (24), 179 (30), 177 (96), 152 (10), 151 (10), 150 (30), 149 (27), 115 (28)
	2.081	276	—	Trace	276 (39), 278 (43), 243 (4), 241 (13), 215 (57), 214 (15), 213 (100), 205 (42), 197 (13), 187 (2), 185 (16), 150 (22), 149 (43), 148 (13), 115 (62)
	2.160	276	—	Trace	276 (39), 278 (39), 243 (13), 241 (20), 226 (5), 215 (56), 214 (11), 213 (100), 205 (51), 187 (16), 185 (17), 178 (34), 177 (50), 150 (24), 149 (62), 115 (70), 114 (35)

[a] Reference = 1-methylnaphthalene.
[b] pH = 3.0, [1-methylnaphthalene] = 2×10^{-5} [total chlorine] = 1×10^{-3}, 96 hr required for 3 half-lives.
[c] pH = 8.0, [1-methylnaphthalene] = 2×10^{-5} [total chlorine] = 1×10^{-3}, 1 hr required for 2 half-lives.
[d] Relative retention time (GC) and mass spectrum identical to those of an authentic standard.
[e] Derivatized with diazomethane prior to GC/MS analysis.

An anticipated difference in reaction kinetics and reaction mechanisms between low and high pH chlorination reactions of PAH has already been noted (*30*). In the present study, therefore, the product distributions for each compound were determined at both pH 3 and pH 8 in order to insure the maximum probability for observing products formed by all reactive chlorine species.

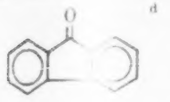
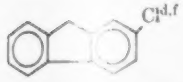
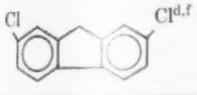
Products

The products observed are listed in Tables 1–8 along with their mass spectral data. In most cases the product distributions were determined after the PAH ($\sim 10^{-5}$ *M*) was allowed to react with $\sim 10^{-3}$ *M* chlorine for five to seven half lives (specific conditions are included in the tables). A small amount of acetonitrile (1 to 8%) was used in many reactions to provide sufficient quantities of PAH products for analysis and to minimize the formation of PAH crystals during a reaction. The use of such small amounts of this organic solvent did not appear to alter the product distribution based on chromatographic comparison with reactions in the absence of acetonitrile. In contrast, reaction solutions containing crystalline PAH material tended to result in the formation of only chloro products rather than both the chloro and oxygenated products observed in homogeneous solutions.

Reactions at high pH tended to produce oxygenated products while those at low pH tended to give both oxygenated and chlorinated products. The predominant product types included mono- and dichlorosubstituted compounds, quinones, phenols, carboxylic acids, and an epoxide. Minor compound types included chlorohydrins and dihydrodiols. A detailed mechanistic investigation of phenanthrene suggested the formation of an arene oxide as the key intermediate in interpreting the product distribution.

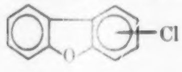
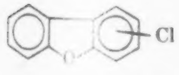
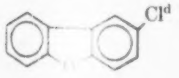
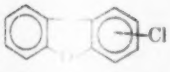
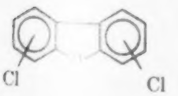
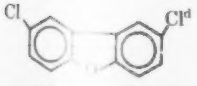
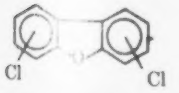
Free-radical substitution on the methyl groups of 1-methylphenanthrene and 1-methylnaphthalene and the benzylic carbon of fluorene was not extensive. This type of reaction which results in the formation of benzyl halides and alcohols has previously been reported for some chlorination reactions (*31*). Benzyl alcohols can be distinguished from ring-substituted

Table 2. Products of fluorene reactions.

Proposed structure	Relative retention time (HPLC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
	0.903	180	—	~ 2%[e]	—
	1.097	200	44%[e]	—	200 (44), 202 (14), 166 (29), 165 (100), 164 (43), 163 (58)
	1.184	234	10%[e]	—	234 (20), 236 (18), 201 (50), 199 (100), 165 (20), 164 (24), 163 (54)

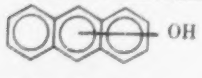
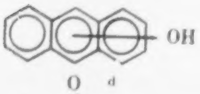
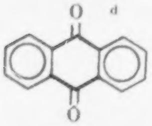
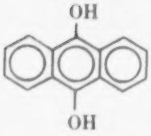
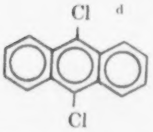
[a] Reference = fluorene.
[b] pH = 3.05 [fluorene] = 1.38×10^{-6}, [total chlorine] = 4.21×10^{-4}, 360 min required for 4.5 half-lives.
[c] pH = 7.70, [fluorene] = 1.38×10^{-6}, [total chlorine] = 4.01×10^{-4}, 7 days required for 2 half-lives.
[d] HPLC retention time and $^{a}254/^{a}280$ identical to those of an authentic standard.
[e] The percent yields were determined by HPLC utilizing authentic compounds as standards.
[f] Relative retention time and mass spectrum identical to those of an authentic standard.

Table 3. Products of dibenzofuran reactions.

Proposed structure	Relative retention time (HPLC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
	1.168	202	Trace	—	202 (100), 204 (50), 175 (6), 173 (10), 167 (7), 139 (61)
	1.190	202	Minor	—	202 (100), 204 (45), 175 (3), 173 (8), 167 (4), 139 (62)
	1.212	202	Major	Major	202 (100), 204 (40), 175 (3), 173 (5), 167 (3), 139 (45)
	1.225	202	Minor	—	202 (100), 204 (45), 175 (3), 176 (3), 167 (2), 139 (43)
	1.389	236	Minor	—	236 (100), 238 (53), 203 (4), 201 (6), 175 (33), 173 (81, 147 (11), 138 (37), 137 (46)
	1.405	236	Major	—	236 (100), 238 (53), 203 (2), 201 (5), 175 (23), 173 (55), 147 (5), 138 (22), 137 (27)
	1.414	236	Minor	—	236 (100), 238 (54), 203 (4), 201 (7), 175 (37), 173 (93), 147 (10), 138 (38), 137 (44)

[a] Reference = dibenzofuran.
[b] pH = 3.04, [dibenzofuran] = 2×10^{-5}, [total chlorine] = 1×10^{-3}, 4 hr required for 4 half-lives.
[c] pH = 8.0, [dibenzofuran] = 2×10^{-5}, [total chlorine] = 1×10^{-3}, less than 50% of starting material reacted in one week.
[d] Relative retention time and mass spectrum are identical to those of an authentic standard.

Table 4. Products of anthracene reactions.

Proposed structure	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present		m/z (relative abundance)
			pH 3[b]	pH 8[c]	
	1.094	194	—	Trace	194 (73), 165 (100), 163 (25), 8[illegible] (31), 62 (34)
	1.123	194	—	Trace	194 (100), 165 (71)
 d	1.182	208	Major	Major	208 (48), 207 (16), 180 (72), 15[illegible] (10), 152 (74), 151 (40), 150 (23), 126 (12) 76 (100), 74 (56)
	1.263	210	Major	Minor	210 (75), 209 (22), 208 (15), 193 (8), 181 (100), 165 (33), 163 (20), 153 (22), 152 (8), 151 (24), 150 (11), 105 (33), 104 (19), 77 (83), 76 (89), 63 (35)
 d	1.317	246	Minor	—	246 (52), 248 (28), 213 (24), 212 (5), 211 (6), 210 (10), 176 (100), 174 (45), 150 (17), 149 (10), 124 (10), 123 (25), 122 (18)

[a] Reference = anthracene.
[b] pH = 3.0, [anthracene] = 1.99 × 10^{-6}, [total chlorine] = 1.0 × 10^{-3}, 30 min were required for 7 half-lives, 4% acetonitrile.
[c] pH = 8.0, [anthracene] = 1.99 × 10^{-6}, [total chlorine] = 1.0 × 10^{-3}, 30 min were required for 7 half-lives, 4% acetonitrile.
[d] Relative retention time and mass spectrum are identical to those of an authentic standard.

Table 5. Products of phenanthrene reactions.

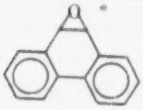
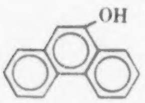
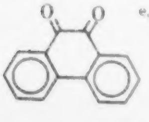
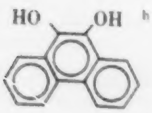
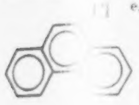
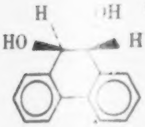
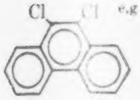
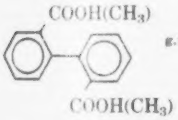
Structure	Relative retention time (HPLC)[a]	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present		m/z (relative abundance)
				pH 3[b,c]	pH 8[c,d]	
 e	0.816	—	194	—	88%[f]	—
 e	0.[illegible]	—	194	19%	~ 2%[f]	—
 e,g	0.726	1.291	208	32%	2%	208 (14), 180 (100), 152 (57), 151 (29), 150 (20)
 h	—	1.080	210	—	Minor	210 (2), 181 (100), 153 (15), 152 (39)

Table 5. *(continued)*

Structure	Relative retention time (HPLC)[a]	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present pH 3[b,c]	 pH 8[c,d]	*m/z* (relative abundance)
 [e,g]	1.128	—	212	34%	—	212 (93), 214 (33), 177 (36), 176 (62), 174 (15), 88 (100), 75 (60)
 [e,g]	0.614	1.235	212	—	3%	212 (20), 194 (22), 181 (62), 166 (66), 165 (100), 163 (11), 152 (25)
 [e,g]	1.251	—	246	7%	—	246 (100), 248 (72), 212 (7), 211 (12), 210 (16), 177 (15), 176 (92), 175 (40), 174 (34)
 [g,i]	—	1.200	270	—	Minor	270 (4), 239 (4), 211 (100), 180 (8)

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[a] Reference = phenanthrene.
[b] pH = 3.03, [phenanthrene] = 8.25×10^{-7}, [total chlorine] = 4.46×10^{-4}, 20 min required for 6 half-lives.
[c] The per cent yields were determined by HPLC utilizing authentic compounds as standards.
[d] pH = 7.70, [phenanthrene] = 6.60×10^{-7}, [total chlorine] = 7.73×10^{-4}, 90 min required for 8 half-lives.
[e] HPLC retention time and A_{254}/A_{280} identical to those of an authentic standard.
[f] Calculated from absorbance data at 254 and 280 nm.
[g] Relative retention time and mass spectrum are identical to those of an authentic standard.
[h] Proposed structure.
[i] Derivatized with diazomethane prior to GC/MS analysis.

Table 6. **Products of 1-methylphenanthrene reactions.**

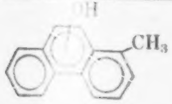
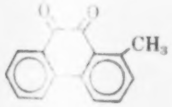
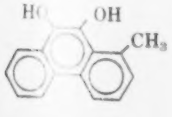
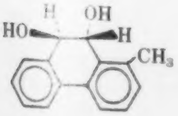
Proposed structure	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present pH 3[b]	 pH 8[c]	*m/z* (relative abundance)
	1.188	208	Minor	Major	208 (100), 207 (22), 180 (19), 179 (39), 178 (44), 165 (68)
	1.197	222	Major	Major	222 (62), 194 (76), 165 (100), 163 (18)
	1.108	224	—	Trace	224 (27), 209 (49), 181 (9), 83 (100)
	1.117	226	—	Major	226 (34), 208 (37), 195 (74), 180 (34), 179 (51), 178 (40), 176 (13), 165 (100), 152 (23)

Table 6. *(continued)*

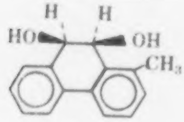
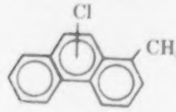
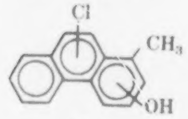
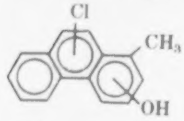
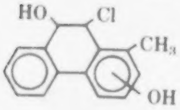
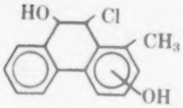
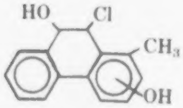
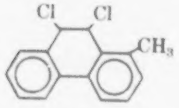
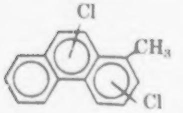
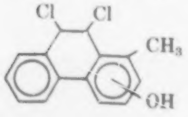
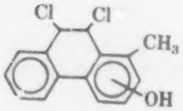
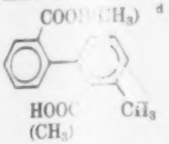
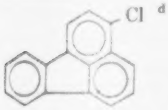
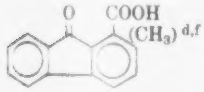
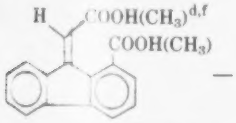
Proposed structure	Relative retention time (GC)[a]	Molecular ion, *m/z*	Amt present		*m/z* (relative abundance)
			pH 3[b]	pH 8[c]	
	1.125	226	—	Minor	226 (50), 209 (20), 208 (63), 207 ([illegible]), 195 (83), 181 (27), 180 (35), 179 (52), 178 (41), 176 (11), 165 (100), 152 (23)
	1.115	226	Minor	—	226 (87), 228 (22), 191 (100), 189 (78), 187 (19), 165 (16), 164 (12), 163 (17)
	1.293	242	Minor	—	242 (92), 244 (26), 207 (33), 179 (100), 178 (70), 176 (28)
	1.305	242	Trace	—	242 (100), 244 (27), 207 (52), 179 (77), 178 (64), 176 (30)
	1.217	260	Minor	—	260 (43), 262 (15), 244 (9), 242 (32), 231 (10), 229 (30), 226 (16), 225 (79), 215 (9), 213 (26), 207 (36), 195 (18), 194 (25), 179 (100), 178 (61), 176 (17), 165 (77), 163 (19), 152 (23)
	1.238	260	Trace	—	260 (60), 262 (22), 244 (14), 242 (47), 231 (21), 229 (68), 225 (5), 215 (9), 213 (21), 207 (55), 195 (15), 194 (27), 179 (100), 178 (67), 176 (21), 165 (81), 163 (25), 152 (22)
	1.246	260	Trace	—	260 (34), 262 (12), 244 (12), 242 (43), 231 (12), 229 (40), 225 (3), 215 (8), 213 (17), 207 (45), 195 (11), 194 (15), 179 (100), 178 (68), 176 (22), 165 (59), 163 (18), 152 (25)
	1.221	260	Major	—	260 (82), 262 (57), 227 (16), 225 (5[illegible]), 190 (61), 189 (100), 187 (34)
	1.230	260	Minor	—	260 (100), 262 (68), 227 (24), 225 (7[illegible]), 190 (32), 189 (64), 187 (21)
	1.174	278	Minor	—	278 (27), 280 (19), 244 (7), 243 (12), 242 (18), 229 (13), 227 (13), 226 (15), 22[illegible] (33), 208 (31), 207 (15), 191 (18), 190 (19), 189 (28), 179 (100), 178 (71), 176 (24), 165 (35), 163 (16), 152 (13)
	1.179	278	Trace	—	278 (43), 280 (31), 245 (35), 244 (26), 242 (36), 226 (16), 191 (19), 190 (18), 189 (27), 179 (34)

Table 6. (continued)

Proposed structure	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present pH 3[b]	Amt present pH 8[c]	m/z (relative abundance)
 [d]	1.006	284	Trace	Major	284 (4), 252 (18), 226 (12), 225 (68), 210 (19), 209 (12), 194 (31), 193 (44), 181 (30), 179 (19), 165 (100)

[a] Reference = 1-methylphenanthrene.
[b] pH = 3.0, [1-methylphenanthrene] = 1.39×10^{-5}, [total chlorine] = 1.0×10^{-3}, 1 hr required for 7 half-lives.
[c] pH = 8.0, [1-methylphenanthrene] = 1.39×10^{-5}, [total chlorine] = 1.0×10^{-3}, 23 hr required for 7 half-live
[d] Treated with diazomethane prior to analysis by GC/MS.

Table 7. Products of fluoranthene reactions.

Proposed structure	Relative retention time (HPLC)[a]	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present pH 3[b]	Amt present pH 8[c]	m/z (relative abundance)
 [d]	1.140	—	236	72%[e]	—	236 (100), 238 (29), 201 (32), 200 (50), 199 (13), 198 (15), 100 (18)
 [d,f]	—	1.064	238	—	Major	238 (29), 208 (18), 207 (75), 180 (69), 179 (48), 152 (28), 151 (72), 150 (56), 75 (100)
 [d,f]	—	1.247	294	—	Minor	294 (19), 236 (20), 235 (100), 220 (40)

[a] Reference = fluoranthene.
[b] pH = 2.99, [fluoranthene] = 3.32×10^{-7}, [total chlorine] = 3.02×10^{-4}, 242 min required for 4 half-lives.
[c] pH = 8.00, [fluoranthene] = 1.29×10^{-6}, [total chlorine] = 1.82×10^{-3}, 19 hr required for 4 half-lives.
[d] Relative retention time and mass spectrum are identical to those of an authentic standard.
[e] Determined by HPLC utilizing an authentic compound as a standard. 7% unreacted fluoranthene.
[f] Derivatized with diazomethane prior to GC/MS analysis.

Table 8. Products of pyrene reactions.

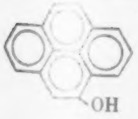
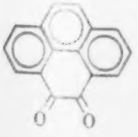
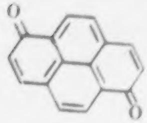
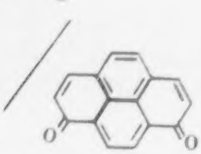
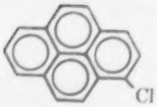
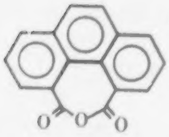
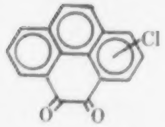
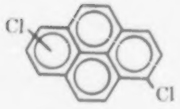
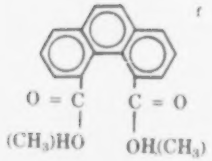
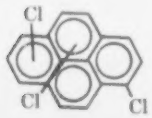
Proposed structure	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present pH 3[b]	Amt present pH 8[c]	m/z (relative abundance)
 [d]	1.178	218	—	Minor	218 (46), 189 (64), 187 (20), 95 (100)
 [d]	1.190	232	Major	Minor	232 (40), 204 (100), 176 (55), 175 (31), 174 (21), 150 (22), 111 (5), 99 (9), 88 (57)

Table 8. *(continued)*

Proposed structure	Relative retention time (GC)[a]	Molecular ion, m/z	Amt present		m/z (relative abundance)
			pH 3[b]	pH 8[c]	
 [e]	1.196/1.20	232	—	Trace	232 (100), 204 (29), 176 (75), 17[illegible] (18), 150 (15), 88 (30)
					232 (100), 204 (25), 176 (70), 17[illegible] (17), 150 (13), 88 (27)
 [d]	1.109	236	Major	Major	236 (14), 238 (12), 201 (59), 200 (77), 100 (100)
 [d]	1.200	248	—	Trace	248 (13), 204 (77), 176 (62), 175 (56), 174 (22), 150 (16), 88 (100)
	1.250	266	Minor	—	266 (6), 268 (3), 240 (8), 238 (23), 212 (4), 210 (15), 176 (40), 175 (69), 174 (70), 99 (41), 98 (46), 87 (100)
	1.172	270	Major	Trace	270 (16), 272 (11), 237 (1), 235 ([illegible]), 200 (47), 198 (25), 100 (100)
 [f]	1.217	294	—	Major	294 (1), 235 (41), 176 (29), 163 ([illegible])
	1.234	304	Trace	—	304 (12), 306 (16), 307 (12), 200 ([illegible]), 199 (48), 74 (10)

[a] Reference = pyrene.

[b] pH = 3.0, [pyrene] = 7.1 × 10^{-6}, [total chlorine] = 1.20 × 10^{-3}, 30 min required for 7 half-lives, 8% acetonitrile.

[c] pH = 8.00, [pyrene] = 6.60 × 10^{-6}, [total chlorine] = 1.10 × 10^{-3}, 90 min required for~4 half-lives, 8% acetonitrile.

[d] Relative retention time and mass spectrum are identical to those of an authentic standard.

[e] Mass spectra and relative retention times were identical to data obtained for an authentic mixture of 1,6-and 1,8- pyrenediones.

[f] Treated with diazomethane prior to GC/MS analysis.

isomers on the basis of prominent M-OH, M-1, M-2 and M-3 peaks in their mass spectra (*32*). In contrast, 1-chloromethylnaphthalene exhibits a mass spectrum which is nearly identical to that of its ring substituted isomer, 1-chloro-4-methylnaphthalene. Fortunately, a difference in relative gas chromatograph (GC) retention time (1.45 versus 1.38, respectively) allowed the conclusion that the former methyl-substituted halide was not produced in either 1-methylnaphthalene experiment. However, it is not possible to ascertain from the present data whether any of the other chloromethyl derivatives of naphthalene or phenanthrene were formed. A hydroxymethyl group is probably present in only one trace product of methylnaphthalene (Table 1, $M^{+\cdot} = 222$).

Biological Implications

An assessment of the biological implications of release of these chlorination products into the environment and into drinking water must, at present, depend upon scattered literature reports of various screening tests on individual compounds. These reports indicate that nearly all of the compound types produced in the chlorination reactions of PAH have the potential for causing adverse biological response. However, it is not possible to accurately predict activity of untested products because the nature of the various substituents and their locations on the aromatic ring may drastically affect the biological activity (*33*).

All of the nonchlorine-containing products of phenanthrene (except diphenic acid) shown in Table 5 have been tested for mutagenic activity by the reversion of histidine-dependent *Salmonella typhimurium* and the rec assay with *Bacillus subtilis* (*34, 35*). The strongest mutagenic activity was observed with phenanthrene-9,10-oxide and 9-phenanthrol, but this activity was much less than that of the control, benzo[a]pyrene-4,5-oxide. Lack of significant mutagenicity and tumorigenicity of the epoxide and *trans*-dihydrodiol of phenanthrene was reported by other laboratories (*36*).

The 1,6- and 1,8-pyrenediones have been reported to be mutagens and enhancing agents to other mutagens (*37*). Anthraquinone shows little or no activity (*38*), but certain derivatives (e.g., phenols) are quite mutagenic (*39*). Quinones of benzo(a)pyrene are mutagenic and the Ames assay without microsomal activation (*40*).

Phenols may be carcinogenic, mutagenic and toxic (*18*) and may bind to DNA, RNA and proteins (*41*). Chlorophenols may also exhibit similar activity (*42*). 2-Naphthol is known to affect the function of renal tubules (*43*), and 1-naphthol is a reported mutagen (*38*).

Chlorinated PAHs (e.g., chlorinated dibenzofurans) may be extremely toxic (*44, 45*). Chloromethyl-(*46*) and hydroxymethyl-substituted PAHs (*47*) may also have mutagenic and carcinogenic activity.

Support for this research was generously provided as a cooperative agreement (CR806892) with the Health Effects Research Laboratory (HERL) -Cincinnati. We also wish to express our appreciation to Dr. F. Kopfler (HERL) for providing helpful guidance during the course of this research. It should also be noted that several preliminary experiments were also performed as part of the Master's thesis of K. J. Welch (1979).

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Environmental Health Perspectives
Vol. 46, pp. 87-99, 1982

Nonvolatile Organic Compounds in Treated Waters

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Over the past decade much information has been published on the analysis of organics extracted from treated water. Certain of these organics have been shown to be by-products of the chlorination disinfection process and to possess harmful effects at high concentrations. This has resulted in increased interest in alternative disinfection processes, particularly ozonation. The data on organics had been largely obtained by using gas chromatography-mass spectrometry, which is only capable of analyzing, at best, 20% of the organics present in treated water. Research in key areas such as mutagenicity testing of water and characterization of chlorination and ozonation by-products has emphasized the need for techniques suitable for analysis of the remaining nonvolatile organics.

Several methods for the isolation of nonvolatile organics have been evaluated and, of these, freeze-drying followed by methanol extraction appears the most suitable. Reverse-phase HPLC was used for separation of the methanol extract, but increased resolution for separation of the complex mixtures present is desirable. In this context, high resolution size exclusion chromatography shows promise. Characterization of separated nonvolatiles is possible by the application of state-of-the-art mass spectrometric techniques.

Results obtained by these techniques have shown that the nonvolatile organic fraction of chlorinated drinking water consists of many discrete compounds. Among these, some of the chlorinated compounds are almost certainly by-products of disinfection. Studies of the by-products of ozonation of fulvic and humic acids isolated from river waters have indicated a similar proportion of nonvolatile organics. Further, ozonation can result in the release of compounds that are trapped in the macromolecules.

Introduction

This paper presents a short review of research into nonvolatile organics in water, with particular emphasis on analytical methodology. In order to limit the size of the paper, the review is necessarily selective in the citation of research from the scientific literature. Results of recent work at the Water Research Centre (WRC) will be used to highlight important areas of research into nonvolatile organic compounds in treated water.

The need for methods of analysis of nonvolatile organics stems from the rapid growth of interest in organics in treated water over the past ten years (*1, 2*). This interest arises from concern over the possible long-term health effects from ingestion of organics in treated waters, particularly halogenated species (*1*). The vast majority of identifications of organic material in treated waters have been generated by the use of gas chromatography-mass spectrometry (GC-MS) techniques, which provide a relatively rapid and straightforward method of analysis. It is now generally accepted that 80–90% of the organic matter present in both raw and treated waters is not amenable to this type of analysis due to insufficient volatility. Further, there is some evidence that a similar proportion of the halogenated organic matter produced by chlorination is also not amenable to GC-MS analysis (*3*). Methods for the characterization of the nonvolatile organics in treated waters are thus urgently needed to give information to complement that obtained from GC-MS. Undoubtedly, concern over possible health effects of the nonvolatile organics, which

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represent the bulk of the organic matter in water, is heightened by the paucity of information concerning them. The rapidly expanding application of short-term bioassay tests, such as the Ames test, to drinking waters is beginning to emphasize this need for qualitative techniques for nonvolatile compounds. Many of the mutagens detected do not seem to be identifiable by GC-MS methods (*4*, *5*).

There are many diverse problems associated with the characterization of nonvolative organics in treated waters, but most of these are a result of the low levels often present, the wide range of compound types and extreme range of molecular weights. Currently there is no analytical method available for nonvolatiles that is comparable in sensitivity and ease of application to the GC-MS approach used for volatiles. It is unlikely that a single method will be sufficient and thus the various steps in the analysis of nonvolatiles are reviewed below.

Isolation Methods

Isolation of a specific compound from a very dilute aqueous solution of a complex mixture of components is a relatively easy process, since the extraction method can be tailored to the chemical and physical properties of that compound. However, isolation of the broadest possible fraction of a complex mixture of unknowns is a considerably more difficult problem. A large variety of methods have been examined for isolation of organics from water, and these have been recently reviewed (*6*). Most of these have been developed specifically for volatile compounds and are, therefore, outside the scope of this paper. Relatively few methods have been developed for nonvolatiles, but several of the methods used for volatiles have been applied, with or without modification.

Research specific to the isolation of nonvolatile organics from water has been mainly concerned with the humic substances. Methods used include adsorption, precipitation and liquid-liquid extraction, and these have been reviewed in some detail (*7*, *8*). Recent research has focused on the use of XAD resin adsorption which has been shown to provide high recoveries (>80%) of humics from raw waters (*9*, *10*). However, XAD is not recommended for the concentration of humic substances after chlorination, as these are very poorly adsorbed (*3*). Other techniques which have been applied to nonvolatile organics in water are carbon–chloroform extraction (*11*), reverse osmosis (*12*) and freeze-drying (*13*, *14*) which suffers form the limitation of being a batch process. Result from these methods suggest that freeze-drying and reverse osmosis provide extracts containing the widest range of compounds. With freeze-drying, the recoveries obtained for particular compound types was found to be solvent dependent, but methanol provided the highest overall recoveries (*14*).

Separation Methods

Separation of nonvolatile organics ideally requires a method that avoids derivatization, does not chemically alter the organics during separation, and has high resolving power and high sensitivity of detection for separated compounds. While no method meets all of these criteria, high performance liquid chromatography (HPLC) approaches this ideal and is the method of choice for the separation of nonvolatile organics.

A variety of fractionation procedures have been used prior to HPLC, particularly where fractions are being prepared for mutagenicity testing. Thus, thin-layer chromatography, column liquid chromatography and chemical fractionation have all been applied for preseparation of nonvolatile organics, and these approaches have been recently reviewed (*5*,*15*). Modern HPLC often involves the use of several chromatographic methods and most of these have been used for separation of nonvolatile organics from treated waters. Size exclusion chromatography (SEC) has shown that the molecular weights of nonvolatiles in water range from a few hundred to >15,000 (*3*,*16*,*17*). One drawback in the characterization of nonvolatile organics according to molecular size by use of SEC is the competition between exclusion processes and nonexclusion processes such as adsorption. This is a particular problem for an unknown mixture of compounds of widely different polarity and can invalidate a molecular weight calibration produced using a mixture of known compounds.

Ion-exchange chromatography has provided high resolution separation of nonvolatiles from chlorinated and ozonated waters prior to identification and mutagenicity testing (*18*,*19*). Reverse-phase HPLC has been applied to the separation of nonvolatiles from treated water to provide fractions for mutagenicity testing (*20*) and mass spectrometric characterization (*13*,*14*). Finally, adsorption HPLC on silica gel G was used to separate neutral organics from a drinking water concentrate prior to mass spectrometry (*21*).

Although HPLC shows inherent advantages for the separation of nonvolatile organics, it suffers from two limitations. There is no universal detector for HPLC which is equivalent in sensitivity to the flame ionization detector used in GC. Several highly sensitive detectors are available, but these are also highly selective and, therefore, inappropriate to the detection of a wide range of compounds. Detection of separated components is usually achieved by ultraviolet absorption and is therefore limited to those compounds containing an ultraviolet chromophore. Secondly, since nonvolatile organic fractions isolated from water usually contain a large number of compounds of diverse chemical type, even high efficiency HPLC columns cannot provide complete separation into individual components. Thus, methods for characterization of HPLC fractions are required that are capable of handling a mixture of components, which is discussed below.

Characterization

Techniques for the characterization of nonvolatile organics can be divided into two broad categories on the basis of approximate molecular weight, each requiring different procedures.

High Molecular Weight Material (>2000). This category consists mostly of humic and fulvic acids—polydisperse polymeric materials, which are not amenable to mass spectral characterization as intact molecules. General methods for their characterization have been extensively reviewed (*8,22*) and only recent work which centers around the production of haloforms and other halogenated organics from chlorination of humic acid fractions (*23–25*) will be discussed.

Halogenated nonvolatile reaction products of humic acids have been characterized by total organohalogen measurement and size exclusion chromatography (*3,26*). The majority of structural studies on aquatic humic materials involve some form of degradation step to produce smaller, volatile compounds for analysis by GC-MS. Included in this approach are attempts to identify the volatile by-products from chlorination (*27*) and ozonation (*28*) of humic materials. The substituted aromatic compounds identified in degradation studies have similar structures to those obtained from soil humic material, suggesting that the two types of humics have similar compositions. Recently, attempts have been made to obtain mass spectrometric data on larger fragments of humic acid (*29*). This has involved field desorption mass spectrometric (FD-MS) analysis of fragments formed after chlorination or permethylation of humic material, but as yet no structural information has been reported.

Low Molecular Weight Compounds (<2000). As mentioned previously, any method used for characterization of nonvolatile organics in HPLC fractions must, in most cases, be capable of working with low levels of individual compounds in complex mixtures. Thus, most of the classical methods of characterization, e.g., nuclear magnetic resonance, infrared and ultraviolet spectrometry, are unsuitable, and necessarily some type of mass spectrometry must be employed. Conventional electron impact mass spectrometry using a direct insertion probe offers a considerable extension to the types of compound that can be handled by GC-MS. However, electron impact suffers from two limitations: the compound of interest has to be volatilized prior to ionization and the molecular ion is frequently of low intensity with respect to fragment ions. The first limitation can be overcome to some extent by the use of rapid heating and "in-beam" techniques, which have been recently reviewed (*30*). The second limitation, however, is difficult to overcome and results in extremely complex, overlapping mass spectra in the case of mixtures.

Ideally then, for mass spectrometry of mixtures of nonvolatiles, a method is required that can ionize compounds in the solid state with transfer of very little excess energy. This would provide a mass spectrum consisting of intense molecular ions with few, if any, interfering fragments ions. Several ionization methods which can achieve this are available, for example: direct chemical ionization (DCI) (*30*), plasma desorption (PD) (*31*), laser desorption (LD) (*32*), field desorption (FD) (*33*) and, most recently, fast atom bombardment (FAB) (*34*). All of these techniques provide intense molecular ions and only limited fragmentation for certain types of nonvolatile organics and are, to some extent, complementary. At the present time, FD-MS has received more extensive application than any of the other techniques and appears to be applicable to the widest range of compound types (*35*). Surprisingly, the use of FD-MS for the characterization of nonvolatile organics separated by HPLC has received only limited study. However, it has been used for analysis of herbicides and their by-products in surface waters (*36,37*) and organics in drinking water (*14,21*). While it has contributed to the identification of only few compounds so far (*13*), it has shown that a complex mixture of nonvolatile organics can be present in potable water but it is impossible to resolve this into individual components even by modern HPLC.

A limitation of FD-MS for the identification of nonvolatiles is the lack of structural information provided. Accurate mass measurement of molecular ions can provide empirical formulae, but a number of structures will still be possible. Recently, methods for obtaining structural data from selected ions, namely, collisional activation (CA) (*38*) coupled with either (B/E) linked scanning (*39*) or mass-analyzed ion kinetic energy spectrometry (MIKES) (*40,41*) have become more widely available. Useful fragmentation information similar to that obtained from electron impact mass spectrometry can be obtained for any ion (of sufficient intensity) in a mass spectrum, which makes the method advantageous for the analysis of mixtures. Reports of the combined use of FD-MS and CA have only recently appeared, but the technique offers great promise for the identification of nonvolatile organics (*42*).

Chemical Studies

The broad spectrum approach to determining the nature and behavior of nonvolatile organics in water has been supplemented by laboratory studies on specific nonvolatile organics known or believed to be present in water. Much of this type of work relates to investigations of the organic by-products formed during chemical disinfection processes such as chlorination and ozonation (*3,18,27,29*). Most studies have been confined, due to a lack of suitable techniques, to products identifiable by GC-MS, i.e., volatile organics. Indirect evidence from such studies, such as results from TOC and TOCl measurement, suggests that a considerable proportion of the by-products are nonvolatile in nature. However, its characterization awaits the development and application of appropriate analytical methods.

The following sections of the paper are devoted to some relevant features of WRC research. In particular, an overall analytical procedure for identification of nonvolatile organics in water, which has been developed recently, is outlined in some detail. Two additional features related to "chemical studies" are included. One involves the laboratory chlorination of uracil. 5-Chlorouracil and other halogenated compounds were detected in treated water in earlier work (*13*). The work reported there was carried out to test the hypothesis that such a compound is formed from uracil (a constituent of sewage effluent) during water treatment chlorination. The other aspect, very much related to the subject of nonvolatiles in water, is the identification of by-products of aqueous ozonation of humic and fulvic acids and some preliminary findings from this work are presented.

Experimental

Isolation

The procedures used for isolation of nonvolatile organics from treated water have been reported in detail elsewhere (*13*). Basically, these involve the freeze-drying of water samples followed by methanol extraction of the recovered freeze-dried solids. Initially, adsorption by XAD was considered as a potential isolation method and in order to provide a comparison between freeze-drying and resin extraction, the following experiments were carried out. Small samples (5 l.) of a treated water were collected; one was acidified (to pH 3 by using ~4*N* HCl), one basified (to pH 10 by using ~4*N* NaOH) and the other left at neutral pH. Each of these samples and a blank consisting of double-distilled water were passed through a resin column (XAD-2), which was subsequently eluted with methanol (~100 ml). The methanol eluates were then concentrated and examined by HPLC as detailed below.

Separation: High Performance Liquid Chromatography

The equipment used consisted of two solvent delivery systems (Waters Associates, Model 6000A), a gradient former (Waters Associates, Model 660; and two ultraviolet absorption detectors operated in series (Cecil Instruments, Model CE 212; LDC, Model 1203), at 280 nm and 254 nm. Syringe injections were made through a stop-flow septumless injection port for analytical separations (up to 10 μl injected) and via an injection valve (Rheodyne Inc., Model 7125) for preparative separations (up to 200 μl injected). Columns (20 cm × 7 mm ID) were packed in our laboratory (*13*) with 5 μm particle size Spherisorb-ODS (Phase Separations Ltd.) and generally had an efficiency of about 18,000 theoretical plates (HETP, 0.01 mm; reduced plate height, 2.2) as measured from a standard mixture of phenols (*43*).

A linear gradient was established from two solvent mixtures consisting of 1% methanol in 0.1% aqeous acetic acid (A) and 90% methanol in 0.1% aqueous acetic acid (B). The gradient was run over 30 min from 0% to 100% B. The flow rate was maintained at 2.0 ml/min.

Size exclusion chromatography (SEC) was carried out on a TSK 3000 SW (Toyo Soda Co.) column (30 cm × 7 mm I.D.) with the use of either water or an aqueous solution of potassium chloride and sodium dihydrogen orthophosphate (0.1*M*) as eluent (1 ml/min). Injection and detection were performed as for reverse-phase chromatography.

Characterization

Preparative HPLC fractions were selected on the basis of a relatively high ultraviolet absorption for mass spectrometric analysis on a VG Micromass ZAB lF instrument. Electron impact was carried out at 70 eV electron voltage, 200 μA trap current, 8 kV accelerating voltage, source temperature of 200°C and a resolution of ~4000. Samples were introduced into the source via an independently heated direct insertion probe. Field desorption mass spectra were obtained on the same instrument, also at 8 kV accelerating voltage and with a total extraction voltage of 10 kV. The FD mass spectra were obtained at a resolution of ~1500, using 10 μm tungsten wires with carbon microneedles (average length ~40 μm) grown in the usual manner (*44*). All mass spectra were acquired and processed on a VG Datasystem computer.

Accurate mass measurements were made on selected peaks using either data system acquisition or peak matching. Collisional activation (CA) studies were performed by admitting the collision gas (helium) into a collision cell (in the first field free region) to pressure sufficient to reduce the intensity of the parent ion beam by 60%. The CA mass spectrum was then obtained by scanning the mass spectrometer at a fixed ratio of B/E and recorded oscillographically.

Chemical Studies

Chlorination of Uracil. A series of laboratory experiments was carried out on the aqueous chlorination of uracil. Distilled water (2 l.), buffered (pH 7.5) with borate, was spiked with uracil (1 and 10 mg/l.) and aqueous hypochlorite added to produce a chlorine residual (12 and 1.2 mg/l.). The solutions were stirred continuously and maintained at ambient temperature (20°C) in a water bath. The experiments were carried out in the dark, and samples were withdrawn at regular intervals for determination of pH, free and total chlorine residuals (DPD method) and 5-chlorouracil production. The latter was monitored by freeze-drying the sample (10 ml), extracting the residue with methanol (3 × 5 ml) and submitting the extract to HPLC. One sample from the experiment carried out at high chlorine residual was extracted with *n*-pentane (2 × 5 ml) and analyzed for haloforms by GC-MS. System blanks were carried out in an identical manner to that above, but without addition of uracil.

Ozonation of Humic and Fulvic Acids. Ozone was prepared from dry oxygen (typically ~15 mg/min at a flow rate of 180 ml/min), using a Gallenkamp GE-150 generator. The output was monitored before each experiment by determining the amount of free iodine liberated from KI solution (*45*). Reactions were performed in a twin-necked round-bottomed flask (500 ml) fitted with a bubbler.

Solutions of fulvic and humic acids (200 mg) extracted from river water as previously described (*46*) were prepared in aqueous 1*M* NaOH (25 ml) and diluted with double-distilled water (175 ml). Solution pH was adjusted to ~6 by addition of aqueous HCl prior to ozonation (1 hr, typically 5 mg O_3/mg fulvic or humic acid). By means of a technique based upon adsorption onto XAD-2 resin (*47*), unozonized and ozonized solutions were extracted after acidification to pH 1 with aqueous HCl. After concentration under reduced pressure (~ 2 ml), the aqueous eluate was examined by HPLC.

The XAD-2 resin column was sequentially eluted with ether (25 ml) and methanol (30 ml). The ether fraction was concentrated (to ~1 ml) by evaporation prior to GC and GC-MS analysis, and the methanol fraction was concentrated (to ~2 ml) under reduced pressure prior to HPLC analysis.

The XAD-2 resin was regenerated using a modified procedure to reduce the amount of organic contaminants released from the resin (*48*). Blank experiments were carried out for all of the ozonations and resulted in chromatograms which were essentially devoid of peaks.

GC was performed on a chromatograph equipped with flame ionization detector (Carlo Erba Fractovap 2150), Grob splitless injector, and a wall-coated OV-1 Pyrex capillary column (25 m). GC-MS was carried out in the electron impact mode (Finnigan 4000) with computerized data acquisition and processing (INCOS 2300). A wall-coated silicone-fluid fused silica capillary column (20 m) was used with a Grob splitless injector.

HPLC equipment used was similar to that outlined earlier in the experimental section consisting of two-solvent delivery systems (Waters Associates, Model 6000 A), a gradient programmer (Water Associates, Model 660), and an ultraviolet absorption detector (LDC Spectromonitor II) operating at 240 nm. A reverse-phase column (25 cm × 4.6 mm I.D.) of C_{18}-bonded silica (DuPont Zorbax ODS) was used with a linear gradient of water/methanol (0% to 100%) containing an ion-pairing reagent (tetrabutyl ammonium bromide: 0.01*M*).

Results and Discussion

Isolation

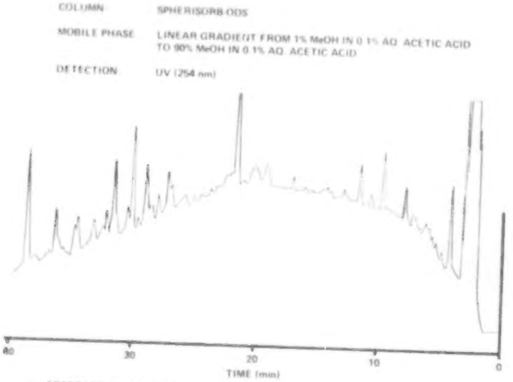
As mentioned above, our studies with model compounds have indicated that freeze-drying and methanol extraction is a very efficient method for isolation of a wide range of nonvolatile organics from drinking water. However, it does suffer from two limitations: (1) only a fixed sample size can be processed in one batch and (2) a proportion of the inorganics in the sample is dissolved in the methanol extract. These problems led us to explore the possibility of using XAD-2 resin as a method for obtaining a nonvolatile organic concentrate that was relatively low in inorganics from a large volume of water sample.

Figure 1 compares the reverse phase HPLC chromatograms derived from a drinking water sample at neutral pH using the freeze-drying/methanol extraction and XAD-2 resin/methanol extraction techniques. The XAD-2 extract exhibits a more pronounced "hump" of unresolved compounds and fewer discrete peaks than the freeze-dried extract.

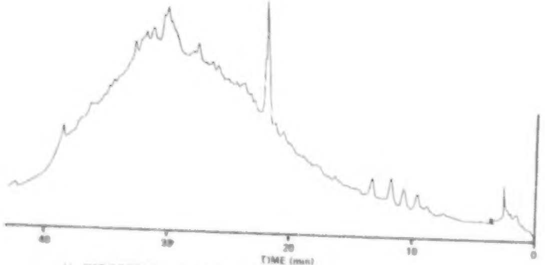
Furthermore, a larger proportion of the XAD-2 extract (eight times) had to be used to obtain a similar ultraviolet response on the chromatograms. The XAD-2 extracts obtained at acidic and basic pH gave chromatograms similar to the neutral pH extract but with fewer discrete peaks. From this comparison of XAD-2 extraction with freeze-drying, it is possible to make the following point with respect to ultraviolet-absorbing compounds present in treated water. XAD-2 resin is less efficient at concentrating nonvolatile organics than freeze-drying and results in an HPLC chromatogram with fewer discrete peaks.

It is probable that this lower efficiency of XAD-2 is to some extent sample-dependent, since the water sample used to compare the different extraction methods was a chlorinated drinking water. It has been suggested (*3*) that the adsorption efficiency of XAD-2 resin for humics is significantly lower for water that has been chlorinated, as compared to the source water.

Freeze-drying and methanol extraction appears to offer the best overall recoveries for a wide range of organics. The major problem with this method remains the significant quantity of inorganics which is dissolved by the methanol. These elute as broad peaks on reverse-phase HPLC and SEC and can interfere with mass spectrometric characterization, which is discussed below. We have evaluated several methods for desalting methanol extracts (*14*), but none of these have been successful in reducing the level of inorganics without adversely affecting the organics. It is possible that the problem will be alleviated by the use of SEC as a further HPLC separation step.



(a) FREEZE DRIED EXTRACT



(b) XAD-2 EXTRACT: 4 µl from 100 µl of MeOH extract

SAMPLE VOLUME: 30 l

FIGURE 1. HPLC profiles of methanol extracts of treated water samples concentrated by (*a*) freeze-drying and (*b*) resin adsorption. Eight times more sample was needed to produce chromatogram (*b*).

Separation

Reverse-phase HPLC is the most suitable method for the separation of complex mixtures of nonvolatile organics isolated from treated water. HPLC chromatograms of treated water extracts consist of a large early eluting peak and many discrete peaks superimposed on a "hump", presumably consisting of unresolved ultraviolet-absorbing compounds (e.g., Fig. 1*a*). Using preparative reverse-phase HPLC, it is possible to separate an organic extract into a large number of fractions for characterization by mass spectrometry. Unfortunately, it is not practicable to examine all of these using the newer methods of MS characterization and it is essential to develop criteria for selection of particular HPLC fractions. Previously, we have selected fractions according to either relatively high ultraviolet absorption and/or relatively high total organohalogen (TOX) values (*14*). However, recent MS evidence (see below) has indicated that the use of TOX levels as a criterion is severely handicapped by the presence of inorganic chlorides. Preparative reverse-phase HPLC fractions that are selected on the basis of high ultraviolet absorption are always found to consist of mixtures of compounds by subsequent MS analysis and only rarely consist of one major compound. This complexity has encouraged us to seek a further separation step in an attempt to reduce the complexity of the mixtures prior to MS characterization. Further separation according to molecular size was an attractive possibility and a high performance size exclusion column was evaluated for this purpose.

The retention volumes and molecular weights of a series of proteins, used to provide a molecular weight calibration, and some model compounds are shown in Table 1. These were obtained on an SEC column using both water (very low ionic strength) and $0.1M$ KCl/NaH_2PO_4 (high ionic strength) as the mobile phase. With water as the mobile phase, an elution order based on molecular size was not obtained. One of the proteins, albumin, was irreversibly adsorbed on the column and the largest

Table 1. Retention volumes on SEC under different elution conditions.

Solute	Mol. wt.	Retention volume, ml[a]	
		H_2O	0.1*M* KCl/NaH_2PO_4
Ferritin	444000	7.0	6.8
Catalase	232000	5.0	8.0
Albumin	67000	NE	8.8
Ribonuclease	13700	ND	10.5
Adenosine diphosphate	550	4.9	11.1
Chlorouridine	278	5.8	11.5
Chlorouracil	146	6.0	11.7
Uracil	112	7.7	12.0
Anisole	108	17.0	ND

[a] ND = not determined; NE = not eluted.

protein, ferritin, eluted after a smaller one, catalase. Further evidence of the occurrence of ionic effects was provided by the small retention volumes of four of the model compounds and the exceptionally high retention volume for the smallest model compound, anisole. Changing the mobile phase to one of higher ionic strength resulted in the expected elution order for the proteins and model compounds based purely on molecular size. The inherently low peak capacity of SEC columns is also demonstrated in that a retention volume of only ~5 ml is available for separation of compounds with a molecular weight spread of ~100 to 500,000. Thus, these columns are appropriate for a crude separation of nonvolatile organics, and are best applied to fractions obtained from reverse-phase HPLC rather than to a total drinking water extract. In order to give some insight into the resolving power of SEC columns for nonvolatile organics of drinking water, a broad reverse-phase HPLC fraction was chromatographed on a SEC column. The chromatogram (Fig. 2) shows two major peaks with some fine structure superimposed suggesting that the extract contains nonvolatile organics of two broad molecular size ranges. It is not possible to determine the molecular size range from this chromatogram, since this is dependent on the nature of the compounds used for calibration of the column which cannot be extrapolated to a complex mixture of unknown composition.

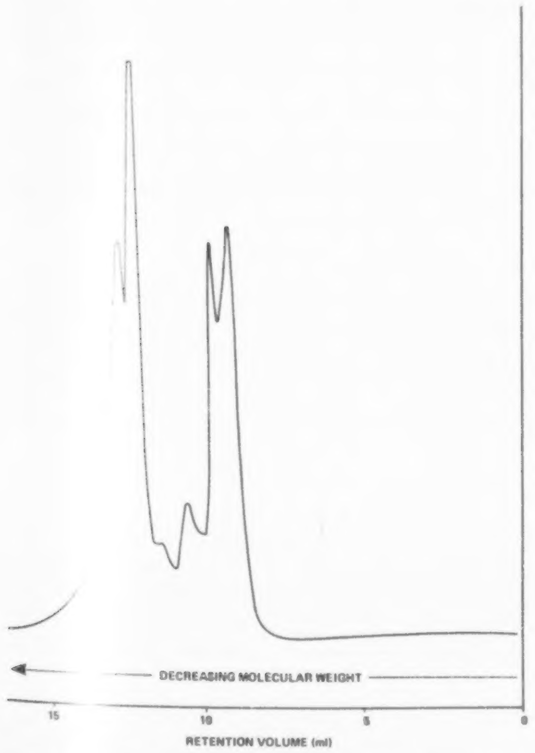


FIGURE 2. Analysis of a reverse-phase HPLC fraction from a drinking water extract using size exclusion chromatography.

Characterization

The results obtained using electron impact (EI) and field desorption (FD) mass spectrometry both show that reverse-phase HPLC fractions of drinking water extracts contain mixtures of compounds with a wide range of molecular weights in agreement with other reports (*21*). An example of a FD-MS of a reverse-phase HPLC fraction of a treated water extract is shown in Figure 3. This represents one of the more complex fractions obtained but illustrates the wide range of compounds present, since the majority of ions can be considered to be molecular ions under the FD-MS conditions used. Identification of these compounds presents many problems and the identifications reported to date (*13*) generally constitute cases where HPLC fractions have contained only one or two major compounds. Halogenated compounds are present, as illustrated by the FD-MS of another HPLC fraction of a treated water extract (Fig. 4). This shows

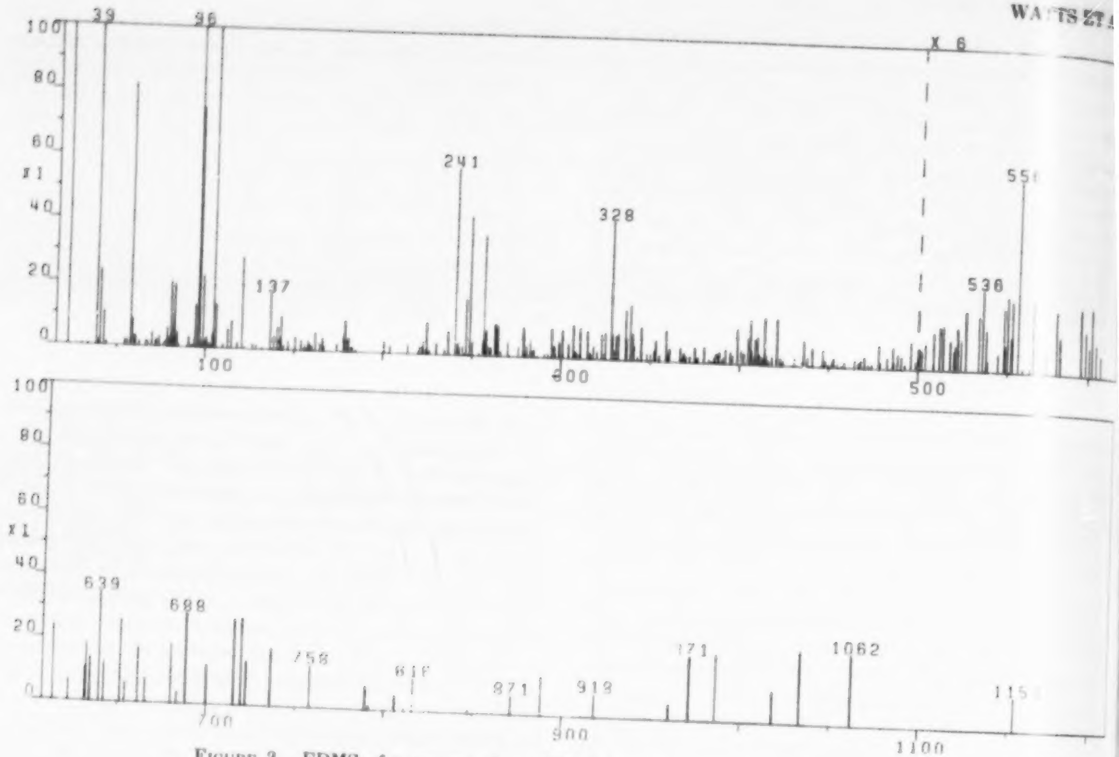


FIGURE 3. FDMS of a reverse-phase HPLC fraction from a treated water extract.

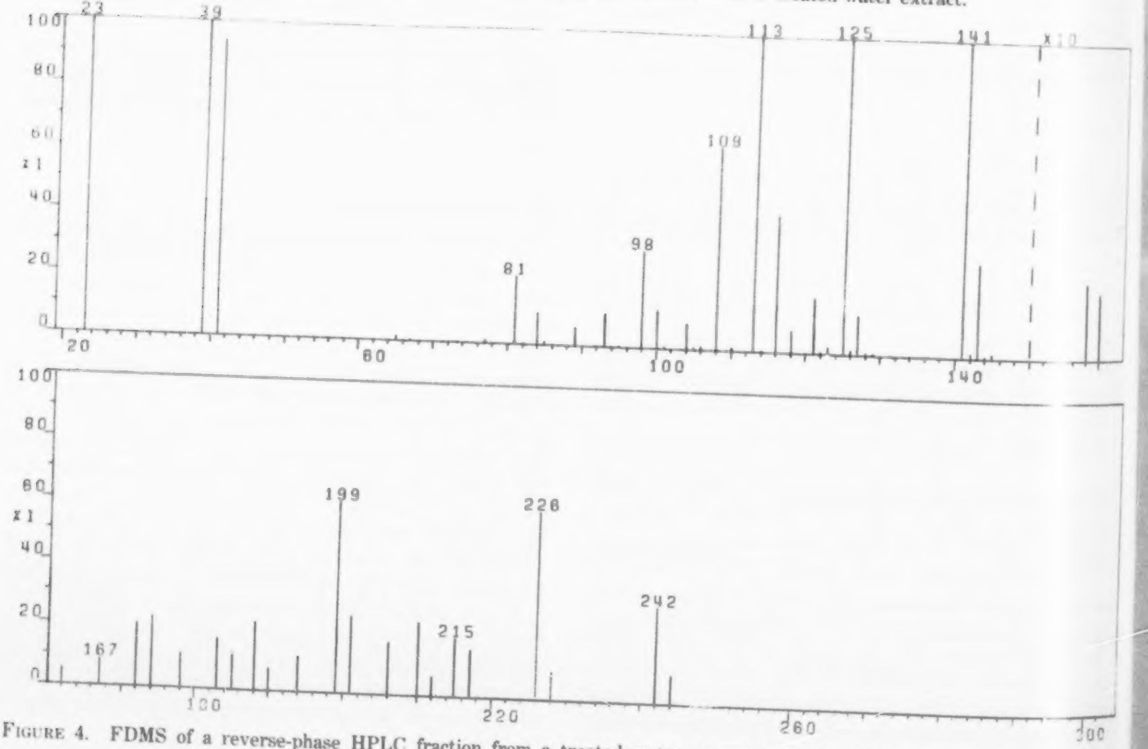


FIGURE 4. FDMS of a reverse-phase HPLC fraction from a treated water extract indicating halogen-containing compounds.

several ions with isotope ratios indicative of compounds which contain chlorine and/or bromine. The methodology we are applying to attempt identification of the nonvolatile organics is shown schematically (Fig. 5).

First, low resolution FD-MS is obtained on a fraction to provide nominal mass data for the organics present and information on the emitter current at which they desorb. Then accurate mass data and hence empirical formulae are obtained on each FD-MS peak of interest by using suitable reference compounds. Either a calibration table is created by the Datasystem which then automatically performs the accurate mass determination or the peak is manually matched against a reference ion and its accurate mass calculated. Structural information is then obtained for each compound by the use of collisional activation and B/E linked scanning. This technique produces the equivalent of a normal electron impact mass spectrum for any molecular ion of sufficient intensity generated by FD-MS.

An example of a FD-CA mass spectrum obtained by linked scanning is shown in Figure 6. The compound used to produce this spectrum was tetrabutylammonium chloride and the FD-CA spectrum was obtained on the tetrabutyl ammonium ion, *m/z* 242. The structure of this ion is clearly indicated by the elimination of methane (*m/z* 226), ethane (*m/z* 212), propane (*m/z* 198) and butane (*m/z* 184). The formation of the tributylammonium ion (*m/z* 185) and similar eliminations from this (e.g., *m/z* 169, elimination of methane) provide further structural information. The combination of this data with the empirical formula information would allow relatively easy identification of this compound.

Application of this approach is still in its early stages, but it is possible to comment on some of the problems that have arisen. FD-MS is a relatively sensitive technique, requiring only a few nanograms of compound, but produces low intensity fluctuating ion beams as compared to EI-MS. In order to carry out collisional activation and linked scanning on a FD ion beam, a minimum of 0.5μ/g of each compound must be deposited on the emitter. Not only does this mean that there is a relatively high (for mass spectrometry) minimum sample requirement, but also if there is a very complex mixture, it may only be possible to examine one or two of the compounds present before exhausting all of the sample. The presence of large amounts of inorganics in methanol extracts of treated water and in some derived HPLC fractions can interfere with FD-MS in several ways; (1) they limit the absolute amount of a fraction that can be deposited on a FD emitter, (2) they can give rise to high molecular weight cluster ions for polar molecules (*49*) and (3) they can yield, by formation of cluster ions, FD-MS spectra with ions at similar molecular weights to compounds of interest, which can complicate interpretation of the mass spectrum (*33*). However, it is likely that the problems due to inorganic constituents will be overcome by either improved HPLC resolution and/or FD-MS handling techniques. Even if these problems cannot be solved, FD-MS with collisional activation and linked scanning is by far the most promising technique available for the identification of nonvolatile organics isolated from water samples.

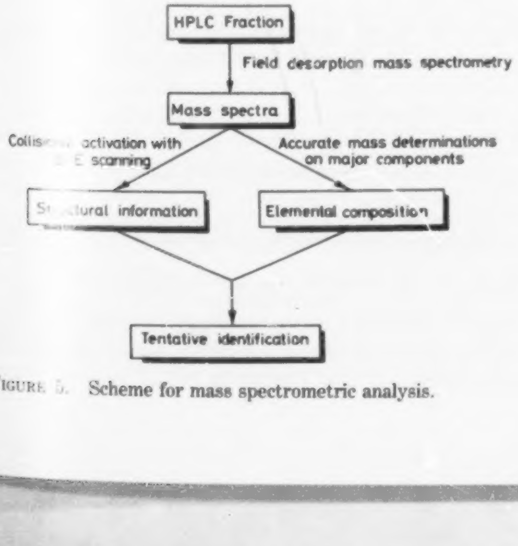

HPLC Fraction
Field desorption mass spectrometry
Mass spectra
Collisional activation with B/E scanning
Accurate mass determinations on major components
Structural information
Elemental composition
Tentative identification

FIGURE 5. Scheme for mass spectrometric analysis.

Chemical Studies

Chlorination. The results obtained from laboratory chlorination of uracil are shown in Table 2. A negligible drop in free chlorine residual of the system blanks was observed during the experiments. GC-MS analysis of a low chlorine residual experiment showed negligible production of haloforms. Several points emerge from study of these results, albeit from a limited number of experiments: (1) the experiment using a high chlorine residual demonstrates that formation of 5-chlorouracil from chlorine and uracil is rapid; (2) 5-chlorouracil is appar-

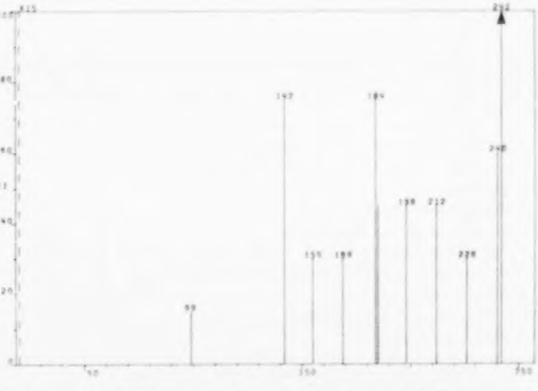


FIGURE 6. FD-CA mass spectrum of tetrabutylammonium chloride.

ently not the ultimate product of the chlorination reaction; and (3) although the ultimate product(s) were not identified, they are not haloforms, at least under the conditions used.

The method used to monitor the course of the reaction did not indicate the appearance of any products other than 5-chlorouracil, hence the end product(s) either does not absorb in the ultraviolet region at 254 or 280 nm or is sufficiently volatile to be lost during freeze-drying or is not extracted into methanol.

Ozonation. The compound types identified by GC-MS of the XAD-2/ether extracts before and after ozonation of humic and fulvic acids are shown in Table 3. Identification is limited to the functional groups that can be recognized from the mass spectral fragmentations, and does not exclude the presence of further oxygenated functionalities in each compound. Full details of the structural assignments will be published at a later date. A typical GC-MS chromatogram of an ether extract of ozonized fulvic acid is shown (Fig. 7) to indicate the complexity of the mixture of volatile organic products.

These results show that the volatile products due to ozonation of humic acid are mainly aromatic, while those of fulvic acid are chiefly aliphatic. Such findings are in agreement with previously published results (*8*). Ozonation of humic acid does not seem to liberate any volatile compounds resulting from fragmentation of the polymer skeleton, although changes are apparent among some of the components detectable after XAD-2 resin extraction of unozonized material. With fulvic acid, ozonation leads to the appearance of certain aromatic acids and normal, branched and possibly cyclic alkanes. While the aromatic acids found in the humic acid extract only after ozonation may result from oxidative cleavage of the macromolecular structure, the alkanes found in the fulvic acid extract only after ozonation were presumably trapped within the polymeric matrix. However, the release of alkanes and DDT and related compounds which are extracted by XAD from humic acid prior to ozonation may be due to a function of the macromolecular structure, which is known to be more "open" at the low pH involved in the XAD extraction procedure (*22*). Estimation of the proportion of volatile organics extracted before and after ozonation suggests that there is little difference for humic acid (< 1%) and only a slight increase (~8% before and ~12

Table 2. Laboratory chlorination of uracil at high and low chlorine residual.

	Time, min	Uracil, concn, µg/l	Cl-uracil concn, µg/l	Chlorine concn, mg	
				Free	Total
High Cl	0	1000	0	12	
	0.5	5	50	11	
	30	5	20	10	
	60	0	0	10	
Low Cl	0	10	0	1.2	1.
	0.5	5	0.5	1.1	1
	15	5	0.5	0.75	0.
	30	4	0.5	0.55	0.
	45	5	0.5	0.4	0.
	50	4	1.0	0.35	0.

Table 3. Compound types present in XAD-2 ether extracts of ozonized and unozonized humic and fulvic aci

Humic acid	Fulvic acid
Alkyl benzenes (chiefly methyl and ethyl)[a]	Hydroxybenzoic acids
Naphthalene and methylnaphthalenes[a]	Aliphatic alcohols (up to ~ C_{20})
Hydroindenes[a]	Aliphatic diols (up to ~ C_{10})[c]
Aromatic acids (1,2,3-ring)	Aliphatic acids (mainly branched up to ~ C_{20})
Aromatic carbonyl compounds[b]	
Substituted phenols (CHO, OMe, COMe, COOH groups)	Aliphatic diacids (C_{2-10})
Heterocyclics (chiefly furans and benzofurans)	Possibly ethers and ketones (aliphatic and mixed aliphatic-aromatic)
Aliphatic acids (n-C_{5-18})	
DDT and derived compounds	Alkanes (normal, branched, ~C_{20-30} and possibly cyclic)[b]
n-Alkanes (n-C_{23-32})	

[a] Present only in unozonized ether extracts.
[b] Present only in ozonized ether extracts.
[c] Probably also includes hydroxy acids, more predominant in ozonized sample.

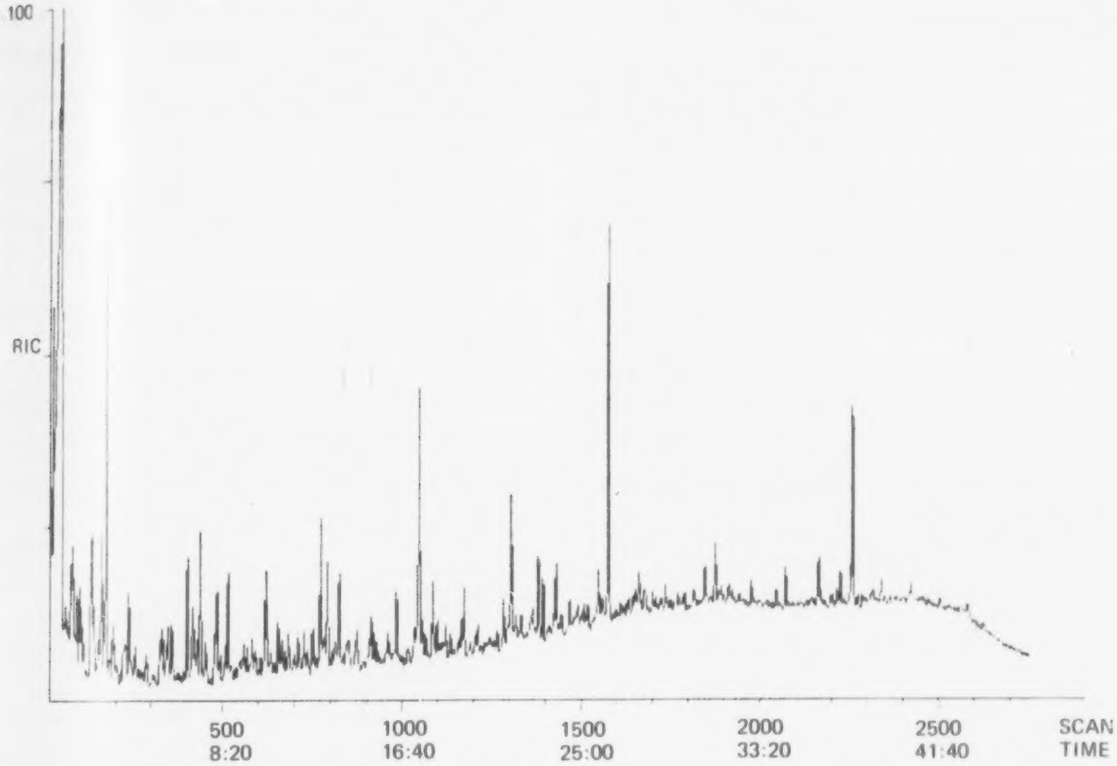


FIGURE 7. GC-MS chromatogram of an XAD-2 ether extract from ozonized fulvic acid.

after) for fulvic acid. Thus, despite the severe conditions of ozonation (~10:1 w/w ozone to humic acid), only a relatively small amount of the humic and fulvic acids was converted to volatile organic compounds.

Ozonation of both humic and fulvic acid resulted in the disappearance of most of the color of the starting solutions, which could suggest that a major structural change occurred. Since no major change was detected in the volatile components, it seems that such changes should be detectable in the nonvolatile components. The reverse-phase ion pair HPLC chromatograms of the XAD-2/methanol extract both before and after ozonation (Fig. 8) of humic acid show similar features, i.e., three major peaks. A method is, therefore, required to provide information on the structural changes undergone by the polymeric matrix of the humic and fulvic acids. One appropriate method is FD-MS (see above), which will be applied to the nonvolatile ozonation products in the near future.

The results of TOC measurements of the aqueous solutions which are applied to the XAD column and the aqueous eluate indicate that the majority of the organic matter is not adsorbed onto the resin column. These water-soluble organics are also nonvolatile in nature and exhibit similar chromatographic behavior to those in the methanol extract of the XAD column. Investigations of these water-soluble organics will be reported at a later date.

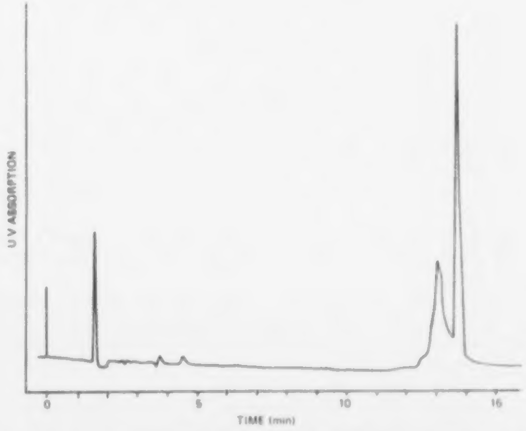


FIGURE 8. Reverse-phase ion pair HPLC chromatogram of an XAD-2/methanol extract of humic acid after ozonation.

Conclusions

Nonvolatile organic compounds account for most of the organic matter present in both raw and treated water. Many of the industrial, agricultural and pharmaceutical compounds in widespread use are nonvolatile and undoubtedly some proportion of them find their way into the aquatic environment. Studies of the organic by-products of chlorination and ozonation of water have shown that a significant proportion of these are nonvolatile. The increasing application of short-term bioassay tests to drinking water has shown that nonvolatile organics could be responsible for some of the mutagenic response.

Until recently there have been no really suitable techniques available for the isolation, separation and characterization of nonvolatile organics from water. Consequently, few data are available on the types and amounts of nonvolatile organics present in drinking water.

Recently there have been significant analytical developments, particularly in mass spectrometry, of direct relevance to nonvolatile organics. New techniques have emerged for the characterization of nonvolatile organics and the approach using freeze-drying, HPLC separation and FD-CA mass spectrometry appears to offer great promise in this respect. Without doubt, the identification of nonvolatile organics in water would benefit considerably from a more widespread application of new techniques such as these.

Much of the work discussed in this paper was funded by the Department of the Environment. Permission from the Department of the Environment and the Water Research Centre to publish this work is gratefully acknowledged.

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Environmental Health Perspectives
Vol. 46, pp. 101-110, 1982

Reaction Products of Chlorine Dioxide

by Alan A. Stevens*

Concern over the presence of trihalomethanes and other chlorinated by-products in chlorine-disinfected drinking water has led to extensive investigations of treatment options for controlling these by-products. Among these treatment options is the use of an alternative disinfectant such as chlorine dioxide. Although chlorine dioxide does not react to produce trihalomethanes, considerable evidence does exist that chlorine dioxide, like chlorine, will produce other organic by-products. The literature describes chlorinated and nonchlorinated derivatives including acids, epoxides, quinones, aldehydes, disulfides, and sulfonic acids that are products of reactions carried out under conditions that are vastly different from those experienced during drinking water treatment. Evidence is beginning to emerge, however, that some by-products in these categories may be produced. Certain specific volatile aldehydes and halogenated derivatives as determined by the total organic halogen parameter are among those by-products that have been measured.

Introduction

The study of organic compounds in drinking water continues to be of intense interest. Extensive work in this area of drinking water research started with the disclosure of the results of a 1974 study of New Orleans drinking water where source water contaminants were still found in treated drinking water (*1*). At about the same time, a different problem was described in two papers: trihalomethanes (chloroform, bromodichloromethane, dibromochloromethane and bromoform) were found in finished drinking waters, but were not present at detectable concentrations in the source water (*2,3*). Indications were that the trihalomethanes were not typically source water contaminants but were formed during the chlorination/disinfection part of the treatment process.

Because of concern as to the significance of formation of chlorinated organic compounds during the water treatment process, the United States Environmental Protection Agency (USEPA) included the measurement of these four trihalomethanes in its National Organics Reconnaissance Survey (NORS) of drinking water from 80 selected cities (*4*). As a result of analysis of source and finished waters in that study, the occurrence of trihalomethanes in finished drinking water was demonstrated to be widespread and a direct result of the chlorination practice. A multitude of other studies has since confirmed these results. Furthermore, natural humic material was demonstrated to be precursor to trihalomethane formation and is present in virtually all source waters, indicating the ubiquity of the problem wherever chlorination is practiced (*5,6*).

Because of findings concerning the carcinogenicity of chloroform and the confirmation of the ubiquity of chloroform and other trihalomethanes in chlorinated drinking water, the USEPA has promulgated a regulation specifying a maximum contaminant level (MCL) of 0.10 mg/l. of total trihalomethanes (arithmetic sum of weight concentrations of $CHCl_3$, $CHBrCl_2$, $CHBr_2Cl$ and $CHBr_3$) (*7*). Utilities must use available knowledge and technology to adjust or change treatment processes to reduce higher concentrations to below the MCL.

Three treatment approaches that can be considered for preventing trihalomethanes from reaching the consumer (*8*) are: (a) remove precursor (humic material) before chlorination, (b) remove trihalomethanes after they are formed, and (c) change disinfectant. This paper concerns the third option only, specifically the use of chlorine dioxide as the alternate disinfectant and focusing on the possible influence of this treatment on the chemical contaminants in the finished water.

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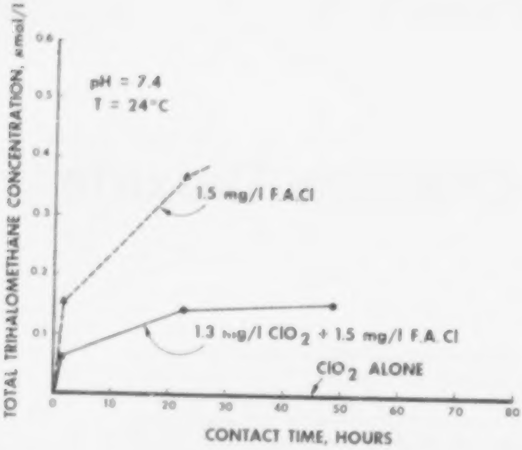

FIGURE 1. Trihalcmethane formation in clarified Ohio River water by chlorine dioxide and excess free available chlorine. Data of Miltner (*9*).

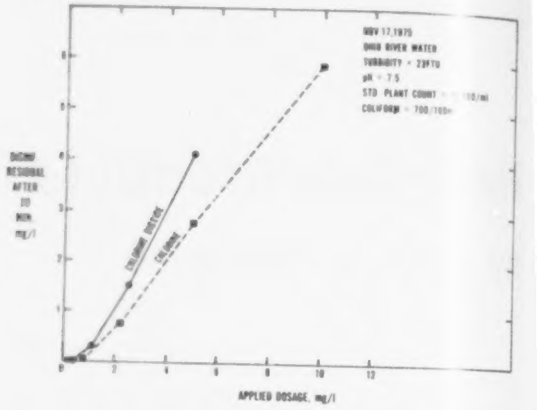

FIGURE 2. Comparison of chlorine dioxide and free chlorine demands for Ohio River water.

Early results of work by Miltner (*9*) to monitor the time dependence of trihalomethane production after chlorine dioxide, chlorine, and chlorine dioxide with chlorine were separately applied to USEPA pilot plant settled water are shown in Figure 1. The upper curve represents the action of chlorine alone; the curve coincident with the abscissa represents the application of chlorine dioxide alone; and the curve between these two represents the action of chlorine to form trihalomethanes in the presence of chlorine dioxide. His results demonstrated two important points: (a) chlorine dioxide did not cause the formation of trihalomethanes at a near neutral treatment pH, although this formation has been reported in one instance to occur at pH 12 (*10*), and (b) chlorine dioxide plus excess chlorine (as is often the case in water treatment) caused formation of lower concentrations of trihalomethanes than the same amount of chlorine alone. Further, other in-house studies, as well as those reported by Roberts et al. (*11*), indicate a yet unexplained decrease in the trihalomethane yield as the ratio of applied ClO_2/Cl_2 increases. Therefore, with consideration only to minimizing trihalomethane formation, chlorine dioxide is a viable alternative to chlorine as a disinfectant in water treatment.

Chlorine dioxide has been widely used in Europe as an alternative to chlorine for drinking water disinfection for some time (*12*). Although these operations are considered successful with regard to disinfection, control of trihalomethanes through the use of chlorine dioxide has not been well documented in most places. The findings reported by Miltner have been borne out, however, at utilities in Hamilton, OH (*13*), Louisville, KY (*14*), Davenport, IA and Peoria, IL (*15*), and Contra Costa, CA (*16*). Consideration must still be given, however, to other by-products that may be formed by chlorine dioxide treatment.

Considerable evidence exists that chlorine dioxide reacts with organic material during water treatment and therefore is likely to produce by-products:

- Because chlorine dioxide is a good disinfectant, some reaction is taking place between the cell components of the organism and the chlorine dioxide.
- Chlorine dioxide is a strong oxidant, and surface waters exhibit a chlorine dioxide demand similar to that for chlorine (Fig. 2) (*8*).
- At applied chlorine dioxide concentrations higher than those in drinking water treatment and under different conditions, identifiable by-products of reactions with various organic materials have been isolated (*17,18*).
- Chlorine dioxide destroys phenolic compounds when the oxidant is used for taste and odor control in water supplies (*19*).
- Most importantly, as was shown earlier, the presence of chlorine dioxide reduces the formation of trihalomethanes by chlorine. This and other evidence obtained by Miltner (*9*) indicates that chlorine dioxide reacts with natural humic acid. This is not at all surprising because chlorine dioxide is used in the paper industry for delignification of wood pulp and is somewhat effective for reducing color in drinking water supplies.

Before an accurate estimate can be made of the relative safety of the two oxidants with regard to the formation of organic by-products, a detailed investigation and identification of the products formed during disinfection with both chlorine and chlorine dioxide is necessary. The purpose of this paper is to consider briefly the possible organic by-products arising from the use of chlorine dioxide as a disinfectant in drinking water treatment, review available data demonstrating the presence of organic by-products, and briefly discuss the formation of inorganic end products unique to the use of chlorine dioxide. The paper is also intended to be an update of the state of knowledge presented earlier elsewhere (*20*). Presenting the reactions of chlorine dioxide in perspective with the reactions of chlorine when both are applied under the same circumstances is informative.

Organic Reactions of Chlorine Dioxide

Although the literature describing the organic reactions of chlorine dioxide is brief, numerous products of oxidation and chlorination by chlorine dioxide are described. However, the majority of this literature describes chlorinated and nonchlorinated derivatives, including acids, epoxides, quinones, aldehydes, disulfides, and sulfonic acids, that are products of reactions carried out under conditions vastly different from those experienced at water treatment plants. This paper discusses only a few reaction types that are known or suspected to be important in water treatment practice on the basis of some experimental evidence. A more complete review of chlorine dioxide chemistry is available elsewhere (*17,18*). The possible reactions with saturated aliphatic hydrocarbons, olefins, amines, and aromatic compounds (especially phenols) are in the group of likely reactions in dilute aqueous solution. In all examples presented for chlorine dioxide reactions, the authors claimed that the applied chlorine dioxide was free of chlorine or hypochlorite when applied.

Reactions with Saturated Aliphatic Hydrocarbons

No evidence exists that either chlorine dioxide or chlorine undergoes reactions with saturated aliphatic hydrocarbons under water treatment conditions. The question of whether or not free chlorine reacts with saturated aliphatic hydrocarbons in aqueous systems to form chlorinated derivatives frequently arises because of the presence of methane in many groundwaters, and the root name "methane" in "trihalomethane" implies to some that such reactions take place. Such reactions of free halogen with aliphatic hydrocarbons require a free radical mechanism, however, and are improbable in aqueous systems where chlorinated derivatives are formed. The trihalomethane formation can be more readily explained by the classical haloform reaction with methyl ketones. However, the classical haloform reaction is not the complete explanation because certain phenol derivatives, notably resorcinol, do react quite readily with aqueous chlorine to produce chloroform. The relationship of these reactions to those of humic materials with chlorine has been under intense investigation (*21*) and has been reported in part by Johnson (*22*). Reactions of humic materials with chlorine dioxide are discussed in more detail below.

Reactions with Alkenes

Chlorine can react with alkenes by addition across the double bond to produce saturated dichloro derivatives. A more likely course in aqueous systems, however, is to produce chlorohydrins by reaction with hypochlorous acid (*23*).

The reactions of chlorine dioxide with alkenes are apparently very complex and produce a host of chlorinated and nonchlorinated products. Methyl oleate is reported to react at the double bond site to produce aldehydes, the epoxide, chlorohydrin, a dichloro derivative, and α-chloro and α-unsaturated ketones. The aldehyde formation seems to be subject to argument: Leopold and Mutton (*24*) reported aldehyde formation by chlorine dioxide cleavage of the double bond in triolein (the triglyceride of oleic acid), and Lindgren and Svahn (*25*) did not find aldehydes after chlorine dioxide reaction with methyl oleate.

Cyclohexene in aqueous mixture with pure chlorine dioxide has been shown by Lindgren and Svahn (*26*) to produce a similar complex mixture: adipic acid, cyclohexene-3-one, 3-chlorocylohexene, 1,2-epoxycyclohexane, *trans*-2-chlorocyclohexanol, 2-chlorocyclohexanone and *trans*-1,2-dichlorocyclohexane. Note that at least the last three of these products are the same as would be expected from reaction of cyclohexene with chlorine.

Reactions with Amines

Aqueous chlorine and chlorine dioxide react very differently with amines. Chlorine reacts with ammonia and primary and secondary amines to produce the well-recognized chloramines by replacement of hydrogen. Tertiary amines are a special case; their reaction with chlorine produces a chloramine and an aldehyde (*23*). Chlorine dioxide, however, does not react with ammonia and reacts only slowly with primary amines. In general, amines produce the respective aldehydes upon reaction with chlorine dioxide in the following order of reactivity: tertiary > secondary >primary. Chlorine dioxide does not react with ammonia to form chloramines.

Reactions with Aromatic Compounds

The best known reactions of aqueous chlorine with aromatic compounds in the water treatment field are those that occur with phenols. Chlorine reacts rapidly with phenol to form 2- or 4-mono-; 2,4- and 2,6-di-, and 2,4,6-trichloro derivatives (*23,27*). These compounds are highly odorous and are slowly decomposed by excess chlorine. Other phenolics and substituted aromatics can also be chlorinated. The formation of odorous chlorophenols by chlorine treatment is one of the chief reasons that chlorine dioxide has been used as an alternative in drinking water disinfection applications (*19*).

Chlorine dioxide as usually used in drinking water treatment does not seem to cause formation of odorous compounds with phenol but, through a complex mechanism, forms quinones and chloroquinones, and when in excess, oxalic and maleic acids (*18*). As with alkenes, chlorine substitution in the products, however, is not entirely absent. Chlorine dioxide treatment of phenols can cause chlorine substitution or ring cleavage or both, depending on the particular phenol reacted and the conditions of the reaction. The chlorinated products formed by phenol reaction with chlorine dioxide will generally be of different structure and are either less odorous or are formed in much lower yield than those formed by phenol reaction with chlorine. In other reactions, chlorine dioxide reacts at pH 4 with vanillin to give the nonchlorinated β-formylmuconic acid monomethyl ester. Vanillyl alcohol reacts at low pH to produce both a chlorinated quinone and a nonchlorinated product of ring cleavage. Veratryl alcohol produces 4,5-dichloroveratrole. This is one of the products expected from chlorination of veratryl alcohol (*18*).

Some of the more detailed investigations of the reactions of chlorine dioxide with phenols have been accomplished by Glabisz and by Paluch at the Polytechnic University, Szczecin, Poland. A generalization for the reaction type (ring cleavage or ring retention with or without chlorine substitution) has been given by Glabisz (*28*). Glabisz states that, at least at concentrations of 1 mg/l. and above, reaction products of phenols form two characteristic groups. The first is the group in which the ring structure is retained and the end products are quinones. This group is made up of *p*-dihydric and monohydric phenols that are not *para*-substituted. The second group is characterized by those phenols that undergo ring cleavage to give carboxylic acids as end products. Examples include *para* alkyl phenols and *ortho* or *meta* dihydric phenols.

In general, monohydric phenols reacting with chlorine dioxide undergo chlorination along with oxidation, and those of the first group form the chloroquinones as well as chlorophenols. Glabisz considers this to be somewhat similar to the reaction of chlorine with these phenols. Although chlorine dioxide tends to favor oxidation over chlorination, the relative amounts of chlorinated versus oxidized products depend on the relative amounts of both chlorine dioxide used and phenols present. Excess chlorine dioxide favors oxidation.

Measured Individual Organic Compounds: EPA Studies

Because of the potential for undesirable by-product formation resulting from chlorine dioxide disinfection of drinking water, an in-house investigation was begun at the USEPA Cincinnati laboratory to determine if by-products of the type predicted by the literature (where reactions described were carried out at generally higher concentrations of chlorine dioxide and substrate) would prevail under drinking water disinfection conditions. This work was carried out in two phases: a search of gas chromatographic data for differences in purgeable compounds found in chlorine dioxide-treated and untreated waters, and development and use of a more elaborate analytical scheme, including evaporation under vacuum and derivatization, to detect products of a more diverse nature, specifically those expected from reactions of phenolic compounds.

Results of this work tend to support some of the general conclusions resulting from extrapolation of information in the literature given above.

Phase 1—Aldehydes. Two classes of compounds (amines and alkenes) were discussed earlier that could yield aldehydes as end products of chlorine dioxide oxidation under the proper reaction conditions. Although these aldehydes could react with chlorite (also present) at low pH to produce acids, these reactions should be slow at water treatment pH values.

These observations suggest that if certain organic substrates are present in a source water, chlorine dioxide treatment should cause an increase in the concentrations of the respective aldehydes. Preliminary results of some of our in-house work indicate that this may be occurring. Care must be taken, however, in interpreting the quantitative aspects of these data. These data were extracted from some early screening studies with chlorine dioxide in which gross treatment changes were sought.

The methods used in this phase are well documented elsewhere and will not be described in detail here. Briefly, the stock solutions of chlorine dioxide were prepared by oxidation of sodium chlorite; chlorine solutions from the pure gas, and standardization of both oxidants were by procedures selected in previous studies by Miltner (*9,29*). GC/MS analysis of reaction mixtures was by the purge-and-trap method exactly as described by Coleman (*30*). Coleman's method limited expected product identifications in this phase to only the most volatile species.

In the first part of the phase 1 study, two gas chromatograph/mass spectrometer (GC/MS) total ion current profiles (TICP) (Fig. 3) were obtained to indicate compounds purged from samples of Ohio River water. One sample had been treated with chlorine dioxide, and the other was untreated. Some compounds appear to be higher in concentration in the treated sample than in the untreated sample, and some new compounds appear in the treated sample. Many of these were identified as aldehydes by their respective mass spectra. Because the chlorinated compounds are present in such large concentrations compared to the aldehydes, apparent changes in aldehyde concentrations can be observed more clearly after additional data manipulation. A fragment ion common to the mass spectra of low molecular weight aldehydes, but not present in the mass spectra of the chlorinated compounds, was selected. The extracted ion current profile (EICP) for this ion, *m/e* 29, was then plotted (Fig. 4) for both treated and untreated river water, and the peaks corresponding to some aldehydes are labeled.

In computerized output GC/MS data, the largest peak in the profile is 100% of full scale, and other peaks are normalized to it. In these EICPs (Fig. 4), peak heights of diethyl ether (which also has an *m/e* 29 fragment) were adjusted to be equivalent, because diethyl ether was assumed not to change in concentration with treatment. The halogenated compounds are not represented in these plots, and the comparatively higher aldehyde peaks in the treated sample are clearly evident.

A similar set of chromatograms was obtained for an untreated source water (Ohio River) and a corresponding chlorinated tap water. The increase in chloroform concentration is obvious from the TICP (Fig. 5). The *m/e* 29 EICP does not show the obvious differences in aldehyde concentrations caused by chlorine dioxide treatment (Fig. 6). Although these samples are not necessarily derived from source water identical to that used in the ClO_2 treatment experiment (above), the results of the chlorination work are typical of those obtained in many replicate chlorination experiments performed over time using Ohio River water as a source.

As mentioned above, caution must be employed before generalizing from these screening studies. If aldehyde formation is considered important, considerably more analytical work is required to obtain

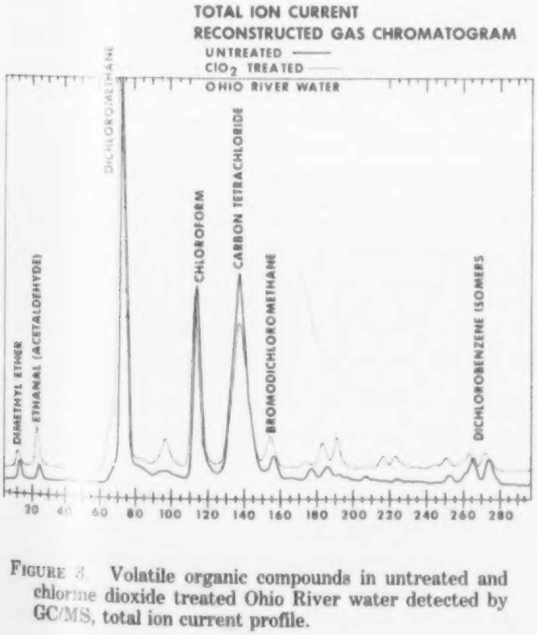

FIGURE 3. Volatile organic compounds in untreated and chlorine dioxide treated Ohio River water detected by GC/MS, total ion current profile.

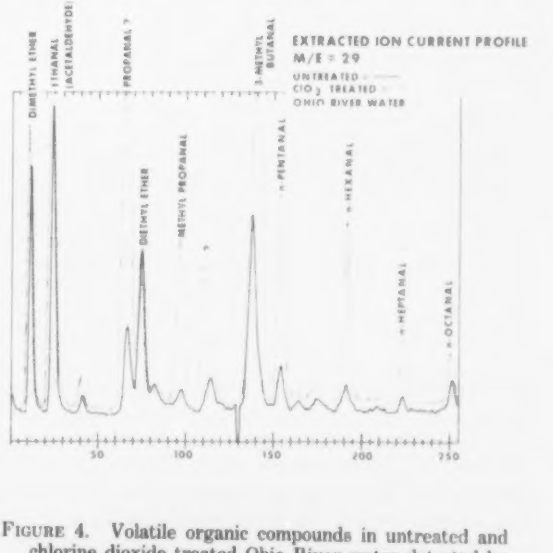

FIGURE 4. Volatile organic compounds in untreated and chlorine dioxide treated Ohio River water detected by GC/MS, *m/e* = 29 extracted ion current output.

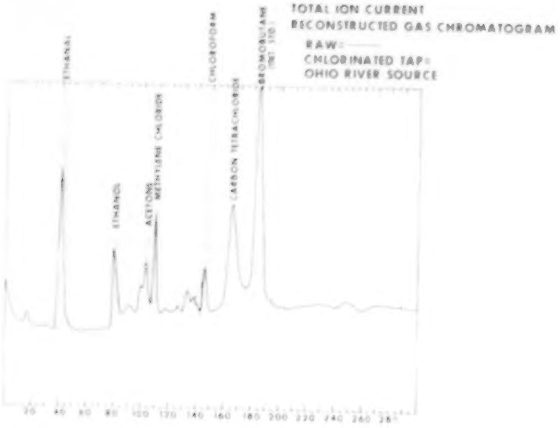

Figure 5. Volatile organic compounds in untreated and free chlorine treated Ohio River water detected by GC/MS, total ion current profile.

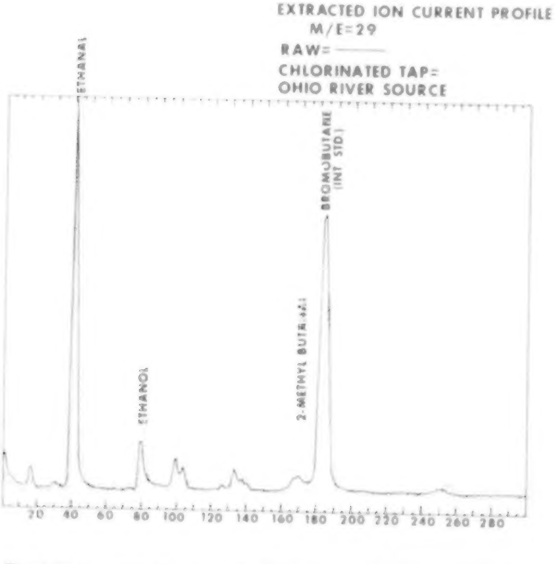

Figure 6. Volatile organic compounds in untreated and free chlorine treated Ohio River water detected by GC/MS, $m/e = 29$ extracted ion current profile.

a good quantitative estimation of these compounds and to adequately assess the magnitude and generality of the observed changes.

Phase 2—By-products from Phenol. Phenol was selected as the model compound for the beginning of the second phase primarily because of the supposed polyphenolic nature of humic materials, which are trihalomethane precursors that make up a large fraction of the organic material present in natural waters where trihalomethane formation is a problem (*31*).

Oxidant solutions were prepared for the second phase work in the same manner as for phase 1. After treatment of the phenol solutions, remaining oxidant residuals were chemically reduced with sodium sulfite. Concentrated ethyl ether extracts were chromatographed after treatment with diazomethane. Routine monitoring of reaction mixtures and quantitation of products was by flame ionization detection. Confirmation of identification was by GC/MS analysis of the same extracts. Spectral interpretation and GC retention times were all confirmed by comparison with authentic standards on the same instruments.

In one experiment, phenol was exposed to varying molar ratios of chlorine dioxide. The data (Table 1) show that chlorophenols were produced at low molar ratios (4/5) of chlorine dioxide to phenol. Higher ratios (14/5 and 14/1) did not produce chlorophenols but favored hydroquinone formation. This product distribution with varying oxidant/substrate ratio was expected to some extent based on the literature (*20,28,32,33*). Although odorous chlorophenolic materials can be avoided in drinking water through the use of chlorine dioxide (*18*), the data suggest that this may not occur if insufficient chlorine dioxide is used. Other expected organic by-products such as oxalic and maleic acids, and 2,6- and 2,5-dichloro-*p*-benzoquinone, discussed earlier, were not immediately identifiable, although total organic carbon concentration data indicated that the phenol was not completely converted to carbon dioxide. To date, no compounds found in the phase 2 experiment nor similar compounds in this category that were not present in the untreated sample have been identified in chlorine dioxide-treated natural waters. Detection limits (calculated as phenol) were estimated to be in the range of 5 to 10 μg/l.

Extensive studies of humic and fulvic acid solutions are currently underway at the University of North Carolina where chlorine dioxide demand and chlorine dioxide degradation experiments are being carried out on aquatic humic materials. Reactions of chlorine dioxide with fulvic acids are apparently slower (or have lower long term oxidant demand) than those of chlorine, and degradation experiments with chlorine dioxide must be carried out initially under conditions designed to "force" the reactions (*34*).

Organic Halogen

Finding individual identifiable species from the chlorine dioxide treatment was not necessarily expected in this work because of the polymeric

Table 1. Products resulting from chlorine dioxide treatment of phenol.

ClO_2/phenol mole/mole[b]	Yield from phenol, %[a]					
	o-Chlorophenol	Phenol (recovered)	2,4-Dichlorophenol	*p*-Chlorophenol	*p*-Hydroquinone	Total recovery
4/5	11	30	0.3	13	3.6	58
14/5	NF[c]	NF	NF	NF	7.2	7.2
14/1	NF	NF	NF	NF	45	45

[a] Reaction time, 4 hr.

[b] In mg/l., 4/5 = 43.5/75; 14/5 = 150/75; 14/1 = 164/16.

[c] None found.

Table 2. Reaction of humic acid with chlorine and chlorine dioxide.

Oxidant/C ratio, mole/mole	Cl_2/ClO_2 doses, mg/l.	Reaction time, hr	$CHCl_3$, μg/l.	Organic halogen, μg/l. as Cl⁻
Cl_2/C				
1/3	3.8	1	39	198
5/3	19.4	1	32	278
ClO_2/C				
1/15	0.75	1	0.4	23
1/3	3.7	2	1.6	52.5

nature of the natural humic material in contrast to the monomeric phenol model. A pyrolysis technique was therefore used to measure the total substituted organic halogen (OX) on these high molecular weight nonvolatile materials (*35–37*). Experimental procedures performed prior to the OX analysis were essentially those referenced or described above. Humic acid solutions were prepared and buffered in the manner described by Stevens et al. (*5,38*). To investigate the possible formation of these higher molecular weight chlorinated species that could not be identified by gas chromatographic techniques, chlorine dioxide was added to humic acid at two different chlorine dioxide to carbon (ClO_2/C) ratios. To compare yields of substituted organic halogen, two reaction ratios with corresponding electron equivalents to chlorine were included in the experiment. That is, the molar ratios 1/15 and 1/3 selected for ClO_2/C correspond to the molar ratios 1/3 and 5/3, respectively, selected for chlorine (Table 2). The basis for this correspondence is that chlorine dioxide reducing to chloride requires five electrons per chlorine atom, whereas chlorine reducing to chloride requires only one electron per chlorine atom. These ratios were selected even though some portions of the chlorine dioxide are typically reduced only to chlorite.

According to the chlorination data (Table 2), with a 1-hr reaction time, the organic halogen yield is much higher than the chloroform yield and increases with chlorine dose, the chloroform concentration remaining essentially constant. Chlorine dioxide produced some (but less) organic halogen and, as expected, an insignificant concentration of chloroform. The trend toward less halogen substitution at the higher ClO_2/C ratio, observed with phenol, was not observed here; this experiment is not definitive, however, because a longer reaction time was allowed at the higher chlorine dose.

Factors influencing organic halogen yields from all disinfectants are currently under careful investigation in the USEPA Cincinnati laboratory. Partial results of these studies at two oxidant doses and 5 mg/l. sodium humate solution are shown in Tables 3 and 4. From Table 3 a general trend to decreasing organic halogen yield with increasing pH at the 10 mg/l. chlorine dioxide dose level at each temperature can be seen. No significant effect of temperature can be seen, probably because these reactions are essentially complete within the 2-hr reaction time (see below). Similar results are shown in Table 4 at the 20 mg/l. chlorine dioxide dose level. Somewhat higher organic halogen yields were observed at the higher chlorine dioxide dose.

In general, the organic halogen by-products of chlorine dioxide treatment were "instantaneously" formed at less than 7% of the maximum formed under similar conditions by chlorine. Concentrations of organic halogen from the chlorine dioxide reaction did not increase with time (up to 160 hr) beyond the initial sampling at 2 hr. This rapid formation of maximum organic halogen concentrations may correspond to the rapid initial oxidant demand reported by Colclough (*34*) also observed in

Table 3. Organic halogen formation with ClO_2 dose of 10 mg/l. to a 5 mg/l. sodium humate solution.

pH	Average organic halogen, µg/l.		
	4°C	20°C	36°C
5.4	26	23	25
7.3	10	10	9
11.5	ND[a]	ND	5[b]

[a] Not detected above blank value.
[b] Two positive of six samples.

Table 4. Organic halogen formation with ClO_2 dose of 20 mg/l. to a 5 mg/l. sodium humate solution.

pH	Average organic halogen, µg/l.		
	6°C	20°C	36°C
5.6	35	40	36
7.5	36	27	25
11.5	8	8	11

Table 5. Inorganic chlorine dioxide by-products.[a]

Species	Initial concentration		Final concentration		ClO_2 demand, %
	mg/l.	mg/l. as Cl^-	mg/l.	mg/l. as Cl^-	
ClO_2	1.5	0.8	0	0	–
ClO_2^-	–	–	0.7	0.4	50
ClO_3^-	–	–	0.4	0.2	25
Cl^-	17.9	17.9	18.1	18.1	25
Totals	–	18.7	–	18.7	100

[a] 1.5 mg/l. ClO_2 added to Ohio River water which was coagulated, settled, dual-media filtered. Contact time was 42 hr; pH was 7.1.

these studies; this is in contrast to chlorine reactions, wherein a steady increase in organic halogen concentrations over a period of days is observed (*39*). In all cases, oxidant residual persisted for the duration of the experiment.

Inorganic By-Products

As noted above, when chlorine dioxide reacts with organic compounds to oxidize them, the by-product chlorite (ClO_2^-) is formed. Furthermore, as chlorine dioxide disproportionates in water, both chlorite and chlorate (ClO_3^-) are formed as by-products. The relative proportion of these by-products was determined during a study reported elsewhere by Miltner (*29*), in which 1.5 mg/l. of chlorine dioxide was added to Ohio River water that had been treated in a pilot plant. The data in Table 5 show that approximately 50% of the original chlorine dioxide was converted to chlorite, about 25% to chlorate, and approximately 25% to chloride. Thus, when chlorine dioxide is used as an alternative disinfectant, the health significance of inorganic anions other than chloride (the sole major inorganic by-product of chlorine treatment) must be considered. These inorganic by-products are unique to chlorine dioxide.

Summary

Inspection of the available literature reveals that a detailed investigation of the aqueous organic chemistry of chlorine dioxide and systematic identification of products formed during water disinfection has not been considered. This must be done before an informed assessment can be made of the relative safety of using chlorine dioxide as a disinfectant alternative to chlorine.

Although trihalomethanes are generally not formed by the action of chlorine dioxide, the products of chlorine dioxide treatment of organic materials are oxidized species, some of which also contain chlorine. The relative amounts of species types may depend on the amount of chlorine dioxide residual maintained and the concentration and nature of the organic material present in the source water. The trend toward lower concentrations of chlorinated by-products with increasing ClO_2 concentration, which was observed with phenols, has not been observed with natural humic materials as measured by the organic halogen parameter. Organic halogen concentrations have been shown to increase with increasing chlorine dioxide dose, but are much lower than those observed when chlorine is applied.

Aldehydes have been detected as apparent by-products of chlorine dioxide oxidation reactions in a surface water that is a drinking water source. Some other nonchlorinated products of chlorine dioxide treatment may be quinones and epoxides. The extent of formation of these moieties within the macromolecular humic structure is also still unknown.

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Environmental Health Perspectives
Vol. 46, pp. 111-116, 1982

Organic *N*–Chloramines: Chemistry and Toxicology

by Frank E. Scully, Jr.[*] and Maxwell A. Bempong[†]

The stability of aqueous solutions of organic *N*-chloramines, suspected of contaminating chlorinated water, has been studied. Two factors influence the decomposition of solutions of *N*-chloropiperidine and *N*-chlorodiethylamine: a spontaneous decomposition and photodecomposition. Since solutions of these compounds are relatively long-lived, a need for an analytical method for their identification is discussed. A new method is described which involves reaction of organic *N*-chloramines with arenesulfinic acid salts. The method gives high yields of stable arenesulfonamides. Several toxicological studies of *N*-chloropiperidine are described. The compound is mutagenic by Ames assay in *Salmonella typhimurium* strain TA 100 and does not require metabolic activation as indicated in a total body fluids analysis using C57BL/J6 mice. *N*-Chloropiperidine was subjected to a modified *in vitro* cell transformation assay using diploid fibroblast cells from Syrian hamster fetuses. A maximum number of foci of 4 per dish was observed at a seeding of 5×10^3 cells/60 mm dish. Under similar conditions, MNNG-induced foci ranged from 4 to 7 per dish.

Introduction

For almost seventy years chlorination of water has been viewed as a public health breakthrough. Recently, however, the observation that some organic by-products of water chlorination produce tumors in laboratory animals (*1,2*) has led to concern about the health effects of water chlorination and more extensive investigation of alternate methods of disinfection.

Many hundreds of organic compounds have been identified in drinking water. However, these represent only a small fraction of the total organic material present.

Routine analysis of residual chlorine in water and wastewater includes an analysis of chloramines, yet no distinction is made between organic and inorganic *N*-chloramines because of the lack of suitable analytical method. Organic *N*-chloramines, as a class, are unusually thermally labile. While some chloramines are distillable, most undergo rapid exothermic decomposition on all attempts at isolation even at room temperature. Some decompose explosively. Therefore, gas chromatographic separation or gas chromatographic/mass spectrometric identification procedures are precluded.

However, organic nitrogen compounds are abundant and essential components of nature. They occur as proteins, amino acids, nucleic acids, and biogenic amines. In addition, free aliphatic amines have been identified in foods including cheese (*3*), fish (*4,5*), beer (*6*), tea (*7*) and milk (*8*).

Among the amines most commonly identified are methylamine, dimethylamine, and ethylamine, as well as three, four, and five carbon aliphatic amines including the heterocyclic amines, piperidine and pyrrolidine. Piperidine and propylamine have been found in drinking water (*9*). Piperidine, pyrrolidine and dimethylamine have been identified in human blood and urine (*10,11*). In fact, Blau has reported that the mean basal excretion in the urine of normal human males over 24 hr is 17 mg of dimethylamine, 15 mg of pyrrolidine, and 5 mg of piperidine.

On chlorination in water, these amino compounds are converted rapidly (*12*) and essentially quantitatively (*13*) to their *N*-chloro derivatives. Therefore a discussion of nitrogenous organic compounds in chlorinated water must be directed toward organic *N*-chloramines and *N*-chloroamino acids rather than free amino compounds. The fact that organic *N*-chloramines have not been identified in chlorinated water raises two questions: are the organic *N*-chloramines which are formed short-lived and, if they are long-lived, how can they be identified? This paper will address these issues.

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In addition we have begun to examine the toxicology of organic *N*-chloramines. The fact that many *N*-chlorodialkylamines are readily synthesized and can be isolated pure makes them attractive candidates for these studies. *N*-Chloropiperidine, the *N*-chloro derivative of an amine that has been found in drinking water, has been the subject of much of our recent work.

Experimental

Stability of Organic *N*-Chloramines

Details of the experiments are described elsewhere (*14*). The aqueous decomposition of *N*-chloropiperidine at pH 7.0 has been repeated at $10^{-3}M$ and $10^{-4}M$ concentrations using a Cary 219 UV/VIS spectrophotometer. The sample chamber was thermostated to 25.0 ± 0.2°C. The instrument was programmed to take an absorbance measurement every 90 min and in the interval remove the sample from the light path. Readings were taken over 60 hr. A plot of ln absorbance versus time gave a straight line of slope $0.0087 \pm 0.0012\ \text{sec}^{-1}$. From this the half-life was calculated.

Derivatization of Organic *N*-Chloramines

N-chloropiperidine was synthesized by the method previously described (*15*). *N*-Chlorodimethylamine and *N*-chlorodiethylamine were synthesized by adding below 5°C the appropriate amine to a slight excess of a standardized commercial solution of sodium hypochlorite (Chlorox) that had been saturrated with salt. After stirring for 10 min, the chloramine was separated from the water in a separatory funnel. *N*-Chlorodimethylamine was purified by drying over freshly activated molecular sieves and distilling on a high vacuum line. *N*-Chlorodiethylamine was dried over anhydrous calcium chloride and distilled at atmospheric pressure. The chloramines were either used immediately or stored in the dark in a freezer.

A solution of 40 mmole of either benzenesulfinic acid sodium salt (Aldrich) or *p*-toluenesulfinic acid sodium salt (Aldrich) in deionized water (100 ml) was stirred in an ice bath. The pure chloramine (20 mmole) was added dropwise over 15 min. Although the sulfonamide formed immediately the product mixtures were routinely stirred overnight to allow all of the sulfonamide to crystallize. The sulfonamides were filtered and dried under vacuum. All three sulfonamides were obtained as white solids requiring no further purification. They each had infrared and NMR spectra, melting points and mixed melting points identical with the same sulfonamides prepared by reaction of the amine with the appropriate arenesulfonyl chloride in basic solution. The yields are found in Table 1.

Table 1. Derivatization of organic *N*-chloramines.

Chloramine	Derivative	Yield, %
N-Chlorodimethylamine	*p*-Toluenesulfonamide	100
N-Chlorodiethylamine	*p*-Toluenesulfonamide	63
N-Chloropiperidine	Benzenesulfonamide	78

Alternatively, amine (20 mmole) and sodium arenesulfinate (40 mmole) were dissolved in deionized water (100 ml). Glacial acetic acid (20 mmole) was added as a buffer and to help dissolve less water soluble amines. The solution was chilled in an ice bath below 10°C and stirred as sodium hypochlorite (30 mmole) was added dropwise over a period of 1 hr. The resulting mixture was stirred overnight at room temperature, filtered, and the sulfonamide dried in a vacuum desiccator. In this manner the benzenesulfonamide derivative of *N*-chloro-*sec*-butylamine was obtained pure in a 95% yield. Similarly, the benzenesulfonamide derivatives of the following *N*-chloramines were obtained pure after recrystallization (pure yield, recrystallization solvent): *N*-chloroaniline (85%, ethanol/water), *N*-chloro-L-leucine (78%, ethanol/water), and *N*-chloroglycine (70%, water). Inorganic chloramine gave an 89% yield of crude benzenesulfonamide which was purified by recrystallization from ethanol/water. *N*-Chloropyrrolidine gave a 100% yield of its *p*-toluenesulfonamide derivative. The *N*,*N*′-bisbenzenesulfonamide derivative of *N*,*N*′-dichloroethylenediamine was prepared in 81% pure yield (recrystallization from ethanol) by the procedure above and the following molar ratios: ethylenediamine (25 mmole), sodium benzenesulfinate (100 mmole), and sodium hypochlorite (100 mmole).

The infrared and NMR spectra, as well as melting points and mixed melting points, were identical in every case to the authentic compound prepared by the reaction of the amine with the appropriate arenesulfonyl chloride in basic solution.

Mutagenicity Studies

Details of the methods used in the toxicity and mutagenicity studies of *N*-chloropiperidine have been described in detail elsewhere (*16*).

Cytogenicity Analysis

Details of the methods used in the cytogenicity studies have been described in detail elsewhere (*16*).

In Vitro Cell Transformation Assay.

The technique of DiPaulo et al. (*17*) was used with primary fibroblast cells from Syrian hamster embryos with the modification described in the text. Details have been described elsewhere by us (*18*).

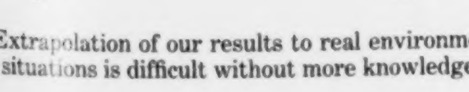
Results and Discussion

Stability of Organic *N*-Chloramines

Recently we reported a study of the aqueous decomposition of *N*-chloropiperidine (NCP) and *N*-chlorodiethylamine (NCDEA) (*14*). The following main points emerged. NCP and NCDEA are considerably longer lived compounds (3.3 days and 2.2 days, respectively, at 25°C at pH 7.0) than the *N*-chloroamino acid, *N*-chloroalanine (55 min), calculated from the data of Stanbro and Smith (*19*). The half-lives of all three compounds do not change from pH 3.5 to pH 9, but increase dramatically below pH 3.0.

Early in our study we found when we followed the NCP concentration by continuously monitoring its absorbance maximum at 262 nm that we photodecomposed it in the sample chamber of the spectrophotometer. Subsequently, special precautions were taken to ensure that photodegradation did not take place in the sample chamber of the UV/VIS spectrometer. To overcome this problem measurements were taken intermittently and the solution removed from the sample beam between measurements. Such a precaution is suggested for all spectrophotometric measurements on chloramines.

The photodecompositions of NCP and NCDEA follow a much different pH profile than their aqueous decomposition. They are most stable at high pH's and photodecomposition rates increase dramatically going from pH 9 to 7 to 5 and lower (Fig. 1). The products of the reaction are imines. NCDEA for instance gave ethylidene ethylamine and acetaldehyde as main products of the photoreaction at pH 7.0 [see Eq. (1)].



(1)

Extrapolation of our results to real environmental situations is difficult without more knowledge of the concentrations of organic *N*-chloramines and *N*-chloroamino acids in chlorinated water. We may conclude that organic *N*-chloramines do undergo a natural first-order decomposition (probably to imines) in water and that their decomposition in the environment is enhanced by ultraviolet light. Finally, under similar conditions of concentration, temperature, and light, *N*-chloramines are much longer lived than *N*-chloroamino acids.

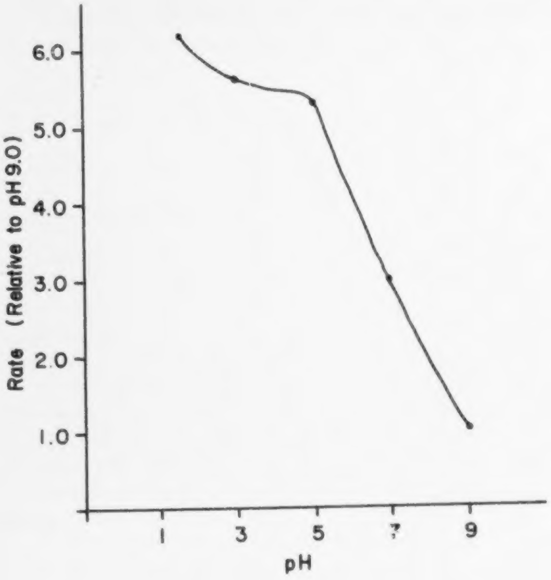


FIGURE 1. Relative rates of photodecomposition of *N*-chlorodiethylamine at varying pH.

Derivatization of Organic *N*-Chloramines

Because of their thermal lability, organic *N*-chloramines are not likely to withstand the harsh conditions of concentration, gas chromatographic separation and mass spectrometric identification. Therefore, if direct isolation is precluded, derivatization is required.

Recently we reported a reaction of organic *N*-chloramines with sodium salts of arenesulfinic acids [Eq. (2)] (*20,21*). The reaction gives good to high yields of stable arenesulfonamides at or below room temperature as shown in Table 1.

$$R_2N{-}Cl + Ph-SO_2^- \longrightarrow Ph-SO_2NR_2 + Cl^- \qquad (2)$$

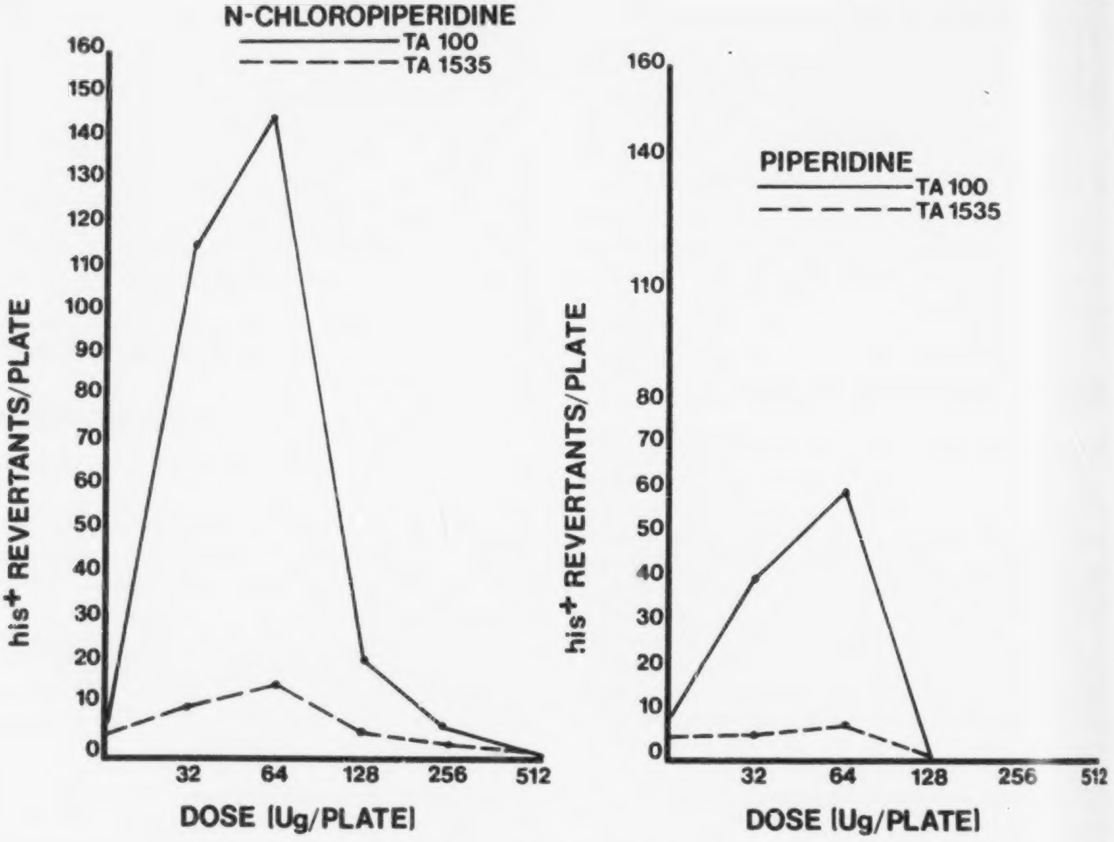


FIGURE 2. Dose-effect relationships for *N*-chloropiperidene and piperidine induction to histidine revertants in TA 100 an TA 1535 Salmonella tester strains.

Many chloramines cannot be distilled and isolated pure the way the three listed in Table 1 can. However, they can be generated in aqueous solution by reaction of the amine with sodium hypochlorite. This can be done either in the presence of the sodium arenesulfinate salt or the chloramine can be generated first and the sulfinate salt added subsequently. In this way, inorganic chloramine (89%) and *N*-chloro derivatives of pyrrolidine (100%), *sec*-butylamine (95%), aniline (85%), and ethylenediamine (81%), as well as others, could be derivatized in good yield. The method is also applicable to the *N*-chloro derivatives of the amino acids L-leucine (78%) and glycine (70%). The examples illustrate the broad scope of the reaction.

Although the derivatizations described here were performed for ease of isolation and product identification on amine or chloramine solutions of approximately 0.2*M*, we are currently extending these studies to dilute solutions.

Toxicological Implications of Chloramine Chemistry

Organic *N*-chloramines have a labile elec rophilic nitrogen which can undergo nucleophilic ibstitution as illustrated, for example, in the deriv ization reaction just discussed. In the classical ork of Miller and Miller, an electrophilic nitrogen is been invoked as the "ultimate carcinogen" res onsible for alkylamination of DNA in the mechanis of the carcinogenic action of aromatic amines (22 It has become clear in recent years that a commo chemical characteristic required for the covalent onding of many "ultimate" chemical carcinogens to DNA is their electrophilic nature. Therefore the emical potential of chloramines suggests the need r more extensive toxicological information on ther .

To date toxicological studies of organic chloramines have been scanty. The comparative lack of bactericidal properties of organic chloramines formed on water disinfection are probably responsible for this, but because of the ubiquity of organic nitrogen compounds in natural water and wastewater as well as in food processing much more is needed.

Mutagenicity Studies

We have reported a study of the toxicity and mutagenicity of *N*-chloropiperidine (NCP) (*16*). NCP is toxic to C57BL/J6 mice with an LD_{50} of approximately 350 mg/kg. In addition, it gave a positive mutagenic response in Ames' *Salmonella typhimurium* strain TA 100 (see Fig. 2). When mice were given a single IP injection of NCP and a total body fluid analysis performed using TA 100 tester strain (see Fig. 2), no metabolic activation of the NCP was detected.

Cytogenetic Analysis

NCP is a strong cytostatic and cytotoxic compound on exposure to Chinese hamster ovary (CHO) cells. It interferes with normal chemical cytogenetic programming of CHO cells resulting in anomalous chromosome separation and nuclear distribution. Macro- and micronucleation were observed. Most importantly, NCP induces structure aberrations of the chromosomes (breaks, exchanges, fragments, ring configurations and centromeric errors), the frequency of which is proportional to the NCP concentration used (*18*).

In Vitro Cell Transformation Assay

Freshly isolated diploid fibroblast cells from Syrian hamster fetuses were used to study *in vitro* carcinogenicity of *N*-chloropiperidine (NCP). Our studies have shown that exposure of early passage cells to NCP (5 – 20 μg/ml) resulted in low cloning efficiency when the cells were plated at 10^3/60 mm dish. The maximum number of foci at this cell density did not exceed 2. We have observed that by using higher concentrations of NCP (1-4 mg/ml) and a higher cell density (5×10^3 to 5×10^4/60 mm dishes) more transformed foci were observed; however, the number of colonies observed in NCP-treated cells was significantly below that observed when *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-exposed cells were analyzed.

The observation that early passage hamster embryo cells do not respond very well to NCP in a transformation assay led us to develop a protocol which seems to increase the number of NCP-induced foci. We have observed that if 12- to 14-week-old hamsters (both male and female) are treated with chronic low doses of NCP *ad libitum*, and if the fetuses of the treated parents are cultured, the number of foci is increased following exposure of the cultured cells to NCP. The maximum number foci per dish originally seeded at 5×10^3/60 mm dish was 4.

The foci were characterized by multilayered, irregularly oriented cells. In the positive control assay, MNNG-induced foci ranged from 4 to 7 per dish.

Conclusion

The fact that *N*-chloropiperidine is mutagenic and transforms mammalian cells *in vitro* is hardly a basis on which to make a health assessment on compounds that have not yet been specifically identified. However, these are the first studies on any members of this class of compounds, which theory suggests contaminate chlorinated water and which should be formed *in situ* on ingestion of chlorinated water. Clearly, more toxicological information is needed on this compound and other members of its class.

As contaminants of chlorinated water, the organic chloramines are obviously considerably shorter lived than, for instance, the trihalomethanes. However, their stability varies with structure and, until actual chloramines formed in water have been identified, a true assessment of the health implications of their presence will be simply speculative.

We are grateful to the U.S. Environmental Protection Agency for support of this under cooperative agreement 807254–01. Dr. F. B. Daniel was the project officer. We are also grateful to Dr. Charles E. Bell for many helpful suggestions concerning the derivatization of chloramines.

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Environmental Health Perspectives
Vol. 46, pp. 117-126, 1982

Toxicology of Organic Drinking Water Contaminants: Trichloromethane, Bromodichloromethane, Dibromochloromethane and Tribromomethane

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This study evaluated the subchronic toxicity of selected halomethanes which are drinking water contaminants. The compounds studied were trichloromethane, bromodichloromethane, dibromochloromethane and tribromomethane. Subchronic 14-day gavage studies were performed with the use of doses encompassing one-tenth the LD_{50} for the compounds. A 90-day gavage study of one of the compounds, trichloromethane, was also done. Parameters observed included body and organ weights, histopathology, hematology, clinical chemistries, and hepatic microsomal enzyme activities. Toxicity to the humoral immune system was assessed by measuring the number of splenic IgM antibody-forming cells and the serum antibody level to sheep erythrocytes. Cell-mediated immunity was evaluated by measuring the delayed type hypersensitivity response and popliteal lymph node proliferation response to sheep red blood cells. The functional activity of the reticuloendothelial system, as measured by the vascular clearance rate and tissue uptake of ^{51}Cr sheep red blood cells was also determined.

The major effects of the halomethanes were increased liver weights, elevations of SGPT and SGOT, decreased spleen weights and a decrease in the number of splenic IgM antibody-forming cells. The humoral immune system appeared to be an indicator of halomethane toxicity. There is evidence that subchronic 14-day exposure may be of greater value than long-term studies in determining the toxicity of these compounds.

Introduction

In a study involving 80 U.S. cities, trichloromethane, bromodichloromethane, dibromochloromethane or tribromomethane were found in all finished drinking water supplies at levels up to 311 μg/l. (*1*). Finished water supplies for which surface water was the original source and in which disinfection was accomplished by chlorination contained the highest levels of halomethanes. Total trihalomethane concentrations were, for the most part, related to the chlorination of raw water containing organic material.

At present the effects of chronic low level ingestion of trihalomethanes in drinking water are not known, although it has been suggested by Cantor (*2*) that an association between bladder cancer mortality rates and trihalomethane levels in drinking water exists. To understand the toxicology of the trihalomethanes better, this study investigated their effects upon the immune system in addition to the usual toxicological parameters. We performed

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subchronic 14-day studies in male and female mice with the selected trihalomethanes, as well as a 90-day study with trichloromethane, to determine if effects seen with short-term exposure would increase or diminish with long-term administration. It was found that the trihalomethanes affected primarily the liver and the spleen, with resulting implications for the immune system, and that certain effects diminished with long-term exposure.

Materials and Methods

CD-1 male and female mice were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts, and housed four per plastic shoebox cage with sawdust bedding and free access to Agway Lab Chow and deionized drinking water. The mice were individually tagged by earpunching and quarantined for one week prior to experimentation. The room was maintained at 21–24°C and a relative humidity of 40–60%. The light-dark cycle was set on 12 hr intervals.

Trichloromethane (Fisher Scientific Co., Richmond, Va. 23230; lot #790372), tribromomethane (Aldrich Chemical Co., Milwaukee, Wisc. 53233; lot #13294–2), bromodichloromethane (Pflatz and Bauer Inc., Stamford, Conn. 06902; lot #320355), and dibromochloromethane (Columbia Organic Chemical Co., Columbia, S.C.; lot #020580) were obtained from commercial sources. Solutions for gavage were prepared fresh daily in 10% Emulphor in deionized water, and appropriate concentrations were administered in a volume of 0.01 ml/g body weight to deliver the desired dose. The dose levels chosen for these studies were 50, 125 and 250 mg/kg/day for 14 days. A 90-day subchronic study was also done for one of the chemicals, trichloromethane, using the same dosages.

Animals were anesthetized with chloroform and blood was collected immediately by cardiac puncture. Gross pathological examinations were performed on all mice. The organs (brain, liver, lungs, spleen, thymus, kidneys, and testes) were then removed, trimmed, and weighed. Material for histologic analysis (kidney, liver, spleen) was fixed in 10% buffered formalin, infiltrated and embedded in paraffin, sectioned at 6 microns, and stained with hematoxylin and eosin. Livers and spleens not destined for histologic study were used in additional procedures.

Blood samples for hematological study were taken in 3.2% sodium citrate. Leukocyte, erythrocyte and platelet counts were performed on a Coulter Counter, Model ZBI. Hematocrits were performed with microhematocrit equipment and hemoglobins determined as cyanomethemoglobin. Leukocyte differentials were evaluated using the standard Wright's-Giemsa staining procedure. Bone marrow cells were flushed from the femur in α-MEM with 5% fetal calf serum and enumerated on the Coulter Counter.

The plasma from the blood samples was assayed for extrinsic activity by prothrombin time. Reagents for this assay were obtained from General Diagnostics. Fibrinogen levels were determined by the kinetic method, using reagents from Dade Diagnostics Inc., Miami, Florida.

For clinical chemistry studies, additional blood samples were drawn by cardiac puncture from animals used for studies on humoral immunity, then allowed to clot. The sera from these samples were loaded onto an Abbott Bichromatic Analyzer, Model 100, and processed according to procedures described in the Operator's Manual.

Livers for microsomal assay were removed, weighed, rinsed, and homogenized at 4°C in four volumes of 0.15*M* potassium chloride containing 0.1*M* potassium phosphate buffer, pH 7.4. The homogenate was centrifuged at 9000*g* for 20 min and the supernatant recentrifuged at 100,000*g* for 1 hr in a Beckman Model L5-50 ultracentrifuge. The microsomes in the pellet were resuspended in 0.05*M* potassium phosphate buffer, pH 7.4 (0.5 g of liver/ml), and aliquots were taken for immediate study.

Microsomal protein was assayed by the method of Lowry et al. (*3*). The cytochrome P-450 content was determined from the reduced CO versus reduced difference spectrum, using an extinction coefficient of 91 $cm^{-1}mm^{-1}$ (*4*). Aminopyrine *N*-demethylase activity was determined by measuring formaldehyde production by the Nash reaction as described by Cochin and Axelrod (*5*). Aniline hydroxylase activity was measured as *p*-aminophenol production (*6*).

The primary IgM response to sheep erythrocytes (sRBC), a measure of humoral immunity, was estimated by the hemolytic plaque assay of Jerne and Nordin (*7*) as modified by Cunningham and Szenberg (*8*). Mice were immunized with 4×10^8 sRBC by IP injection 4 days prior to sacrifice. Spleen cell suspensions were prepared in RPMI 1640 culture medium using stainless steel mesh screens and adjusted to a cell concentration of 10^6/ml for assay of antibody-forming cells (AFC).

As a complement to the plaque assay, the plasma antibody titer was measured by the hemagglutination technique. Seven days after IP injection of 10^9 sRBC, blood was collected by cardiac puncture from chloroform-anesthetized animals into 3.2% sodium citrate. After centrifugation, the plasma was heat inactivated, and serial (1:1) dilutions were made in phosphate-buffered saline. To each of the dilutions in a microtiter well, an equal volume of a 0.5% suspension of sRBC was added. After incubation for 2 hr at 37°C, the plates were observed on a magnifying mirror for agglutination of the sRBC. The antibody titers were expressed as $\log_2$ of the reciprocal of the first dilution with no visible agglutination.

Cell-mediated immunity was evaluated by measuring the delayed type hypersensitivity (DTH) response to sRBC. This is a modification of the methods of Lagrange et al. (*9*) and Paranjpe and Boone (*10*). Sensitization was accomplished by injecting 10^8 sRBC in a volume of 0.02 ml into the left footpad (LFP). Four days following sensitization, the mice were challenged in the LFP with 4 × 10^8 sRBC in a volume of 0.04 ml. At 17 hr following challenge, the mice were injected intravenously with 0.3 ml of ^{125}I-human serum albumin (HSA) (80,000 cpm/0.1 ml). Two hours later, the mice were sacrificed by cervical dislocation, and both hind feet were removed at the ankle joint and radioassayed in a gamma counter. The right footpad (RFP) served as an unchallenged control for background ^{125}I-HSA. A group of mice which was not sensitized, but was challenged as above, acted as unsensitized controls to determine nonspecific swelling. Results are expressed as a stimulation index (SI), which is calculated as follows:

$$\mathrm{SI} = \left(\frac{\text{cpm LFP sensitized}}{\text{cpm RFP sensitized}}\right) - \left[\underline{\bar{\mathrm{x}}}\left(\frac{\text{cpm LFP unsensitized}}{\text{cpm RFP unsensitized}}\right)\right]$$

Popliteal lymph node proliferation in response to sRBC was determined to further evaluate cellular immunity. Mice were sensitized 4 days prior to the last day of gavage by injection of 1 × 10^8 sRBC in 0.02 ml of Alsever's solution into the left hind footpad (LFP). One group of control mice was injected with saline to measure nonspecific stimulation. Four days later, the animals were challenged by injecting 4 × 10^8 sRBC in 0.04 ml Alsever's solution into the LFP. At 1.5 hr after challenge, the mice were given an IP injection of 0.2 ml FUdR (8 × 10^{-6} M). An IV injection of 2.0 μCi ^{125}I-labeled iododeoxyuridine in 0.2 ml saline was administered 30 min later. The mice were sacrificed 20 hr after labeling, and the popliteal lymph nodes were removed and counted in a gamma counter. The stimulation index was calculated as shown below:

$$\mathrm{SI} = \left(\overset{\text{SENSITIZED}}{\frac{\text{cpm left popliteal node}}{\text{cpm right popliteal node}}}\right) - \left[\underline{\bar{\mathrm{x}}}\left(\overset{\text{UNSENSITIZED}}{\frac{\text{cpm left popliteal node}}{\text{cpm right popliteal node}}}\right)\right]$$

Several particles for measuring the phagocytic activity of the fixed macrophage system had been compared previously (*11*). For these studies we used sRBC. Freshly-drawn sRBC (5 × 10^9 cells/ml) were radiolabeled with ^{51}sodium chromate in a 37°C shaker bath with 1 mCi/5 ml cells for 30 min. After chromation, the sRBC were washed with Alsever's solution until the supernatant was virtually radioactivity free. Unlabeled sRBC (5 × 10^9 ml) were added to the labeled cells until the hematocrit was 10%. The resulting cpm's were approximately 200,000/0.1 ml. The sRBC were refrigerated and used the following day. Before use, the cells were washed to remove any free chromium released overnight.

Mice used for evaluation of RES activity were weighed and placed in shoebox cages maintained at 21°C. At zero time, 0.1 ml of labeled particle/10 g body weight was injected intravenously. Blood samples (10 μl) were taken from the tip of the tail at 2, 4, 6, 8, 10, and 15 min. The blood samples were put into 1 ml of distilled water and radioassayed. At the end of 60 min, the mice were sacrificed by decapitation and drained of blood. The liver, spleen, lungs, thymus, and kidneys were removed, weighed, and placed in tubes for counting in a gamma counter. Blood clearance is expressed as the phagocytic index, which is determined by the slope of the clearance curve. Organ distribution is expressed as percent organ uptake and cpm/mg tissue (specific activity).

In order to determine whether or not tolerance to certain halomethanes could be induced, the following experiment was conducted. Groups of male and female mice were gavaged with trichloromethane at the doses previously stated for 90 days. They were then given a single gavaged dose of 1000 mg/kg, an amount approximating the LD_{50} dose, and observed for lethality.

If a one-way analysis of variance of the means showed treatment effects, a Dunnett's *T*–test was performed (*12*). Values which differ from vehicle control at $p < 0.05$ are noted in the tables. Each of the values is given as the mean ± SE of the mean (SEM).

Results

Subchronic Fourteen-Day Studies

CD-1 male and female mice were given trichloromethane, bromodichloromethane, dibromochloromethane and tribromomethane by gavage at 50, 125 or 250 mg/kg/day for 14 days. No chemically induced deaths were noted with any of the compounds. At the end of this period the animals were necropsied. Table 1 lists all the parameters observed in the subchronic 14- and 90-day studies. The data pre-

Table 1. General toxicological and immunological parameters observed following halomethane exposure.

Body weights

Organ weights
  Brain
  Liver
  Spleen
  Lungs
  Thymus
  Kidneys
  Testes

Hematology
  WBC
  RBC
  HCT
  HGB
  Platelets

Hexobarbital sleeping time
Bone marrow
  DNA synthesis
  Stem cell culture
Cell-mediated immunity
  Delayed type hypersensitivity
  Popliteal lymph node proliferation
  Functional status of RES

Clinical chemistry
  Calcium
  Sodium
  Chloride
  Potassium
  Protein
  Glucose
  Cholesterol
  Bilirubin
  Creatinine
  BUN
  LDH
  SGPT
  SGOT
  ALP

Hepatic microsomal activities
  Protein content
  Glutathione
  Cytochrome P-450
  Cytochrome b_5
  Aminopyrine N-demethylase
  Aniline hydroxylase
Humoral immunity
  Enumeration of antibody-forming cells
  Hemagglutination

Table 2. Effects of trichloromethane upon CD-1 male mice following 14 days of exposure by gavage.

Parameter	Vehicle (12)^{a}	50 mg/kg (8)^{a}	125 mg/kg (8)^{a}	250 mg/kg (7)^{a}
Body weights, g	31.3 ± 0.5	29.6 ± 0.5	31.2 ± 0.5	26.3 ± 1.2^{b}
Organ weights				
Liver, mg	1632 ± 48	1734 ± 34	2031 ± 63^{b}	2111 ± 126^{b}
(% body weight)	(5.22)	(5.87)	(6.50)^{b}	(8.01)^{b}
Spleen, mg	141 ± 8	178 ± 7^{b}	149 ± 13	110 ± 6
(% body weight)	(0.45)	(0.60)^{b}	(0.40)	(0.42)
Clinical chemistry				
SGPT, IU/l.	60 ± 9	81 ± 12	95 ± 22	2163 ± 27^{b}
Humoral immunity				
AFC/spleen × 10^5	2.89 ± 0.28	1.44 ± 0.18^{b}	1.35 ± 0.24^{b}	1.02 ± 0.12^{b}
AFC/10^6 cells	3253 ± 171	2057 ± 152^{b}	1700 ± 190^{b}	1405 ± 101^{b}
Hemagglutination ($\log_2$ titer)	7.74 ± 0.23	7.82 ± 0.27	7.32 ± 0.19	7.32 ± 0.22

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's T-test.

sented in this manuscript represents alteration which occurred in a dose-dependent fashion or at the total AFC and at the intermediate and high doses when expressed as AFC/10^6 spleen cells; however, trends are omitted in an effort to minimize presentation of data.

Trichloromethane. Males exposed to the high dose of trichloromethane showed a decrease in body weight (Table 2). Liver weights were increased at 125 and 250 mg/kg regardless of the method of data expression. Spleen weight was significantly elevated at the low dose alone. SGPT was increased in the high dose group. The most striking change occurring in the males was a decrease in AFC expressed as total number and per 10^6 spleen cells at all levels of treatment. Hemagglutination titer was not significantly affected, nor were any alterations in cell-mediated immunity observed.

No significant change in body weight was seen in the female mice (Table 3). Liver weights were increased at all dose levels when the results were expressed as percent of body weight and in the high dose regardless of the method of expression. Spleen weights showed a decreasing trend with increasing dose weight levels but did not attain significance at the $p < 0.05$ level. Changes in clinical chemistry parameters included increased SGOT and SGPT values at the high dose and a decrease in serum glucose at the intermediate and high doses. Humoral immunity was depressed at all doses in terms of highest dose level only. Parameters which were essentially unchanged or did not reveal relevant

Table 3. **Effects of trichloromethane upon CD-1 female mice following 14 days of exposure by gavage.**

Parameter	Vehicle (12)[a]	50 mg/kg (12)[a]	125 mg/kg (8)[a]	250 mg/kg (12)[a]
Body weights, g	26.3 ± 0.6	25.0 ± 0.6	25.2 ± 0.6	25.6 ± 0.8
Organ weights				
Liver, mg	1442 ± 44	1518 ± 27	1517 ± 57	1786 ± 52[b]
(% body weight)	(5.49)	(6.08)[b]	(6.03)[b]	(6.99)[b]
Spleen, mg	151 ± 9	138 ± 8	140 ± 14	134 ± 6
(% body weight)	(0.57)	(0.56)	(0.55)	(0.52)
Clinical chemistry				
SGPT, IU/l.	53 ± 5	47 ± 4	58 ± 4	154 ± 30[b]
SGOT, IU/l.	97 ± 7	78 ± 5	76 ± 4	143 ± 15[b]
Glucose, mg-%	133 ± 4	136 ± 3	114 ± 4[b]	88 ± 6[b]
Humoral immunity				
AFC/spleen × 10^5	1.35 ± 0.3	0.89 ± 0.05[b]	0.58 ± 0.6[b]	0.41 ± 0.05[b]
AFC/10^6 cells	1692 ± 67	1661 ± 84	1157 ± 87[b]	1253 ± 76[b]
Hemagglutination ($\log_2$ titer)	8.07 ± 0.22	7.70 ± 0.18	7.82 ± 0.19	7.40 ± 0.26

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.

Table 4. **Effects of bromodichloromethane upon CD-1 male mice following 14 days of exposure by gavage.**

Parameter	Vehicle (12)[a]	50 mg/kg (8)[a]	125 mg/kg (8)[a]	250 mg/kg (9)[a]
Body weights, g	32.9 ± 0.6	33.2 ± 0.6	31.1 ± 1.0	25.8 ± 1.2[b]
Organ weights				
Liver, mg	1904 ± 65	2050 ± 72	2083 ± 115	2851 ± 79
(% body weight)	(5.78)	(6.17)	(6.70)[b]	(7.21)[b]
Spleen, mg	160 ± 12	143 ± 6	136 ± 10	99 ± 11[b]
(% body weight)	(0.49)	(0.43)	(0.44)	(0.38)
Hematology				
Fibrinogen (mg %)	320 ± 14	345 ± 15	283 ± 10	276 ± 17[b]
Clinical chemistry				
Glucose, mg-%	179 ± 6	197 ± 9	186 ± 16	124 ± 11[b]
SGOT, IU/l.	70 ± 10	159 ± 28	172 ± 19	643 ± 125[b]
SGPT, IU/l.	35 ± 2	91 ± 17	118 ± 29	703 ± 12[b]
BUN, mg-%	23 ± 1	24 ± 2	28 ± 4	47 ± 4[b]
Humoral immunity				
AFC/spleen × 10^5	1.36 ± 0.12	0.96 ± 0.14	0.98 ± 0.13	0.66 ± 0.11[b]
AFC/10^6 cells	1196 ± 85	961 ± 60	1000 ± 78	1024 ± 72
Hemagglutination ($\log_2$ titer)	9.50 ± 0.18	9.20 ± 0.13	8.95 ± 0.18[b]	8.77 ± 0.24[b]

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.

hemagglutination titer was not significantly affected.

Bromodichloromethane. A decrease in body weight was seen in the high dose group of male mice after 14 days of bromodichloromethane administration (Table 4). Liver weights were increased at the intermediate and high dose levels when the data were expressed as percent of body weight. The decrease in spleen weight was significant only at the high dose, but a nonsignificant decrease could be observed at all dose levels. A decrease in the fibrinogen level at 250 mg/kg was seen. Clinical chemistry changes occurred only in the high dose group and included a decrease in glucose and increases in SGOT, SGPT and BUN. The immune system was affected as evidenced by decreases in AFC/spleen and hemagglutination titer, both in the high dose group animals.

Female mice in the high dose group showed a significant reduction in body weight (Table 5). An increase in liver size was manifest in the intermediate and high dose groups only when the data were expressed as percent of body weight. Spleen weight was decreased at the 125 and 250 mg/kg dosages, both as total milligrams and as percent of body weight. Hematological studies revealed a decrease in fibrinogen levels at the intermediate and high dose regimens. Changes in clinical chemistry parameters were observed in the high dose group and included elevations in SGOT, SGPT, and BUN serum levels. The humoral immunity alterations seen as a result of bromodichloromethane administration were decreases in AFC/spleen and hemagglutination titer, observed in the intermediate and high dose groups.

Dibromochloromethane. Fourteen-day administration of dibromochloromethane caused a reduc-

Table 5. Effects of bromodichloromethane upon CD-1 female mice following 14 days of exposure by gavage.

Parameter	Vehicle (12)[a]	50 mg/kg (8)[a]	125 mg/kg (8)[a]	250 mg/kg (11)[a]
Body weights, g	26.6 ± 0.4	26.0 ± 0.6	24.9 ± 0.6	21.5 ± 1.0[b]
Organ weights				
Liver, mg	1432 ± 46	1584 ± 73	1608 ± 59	1553 ± 58
(% body weight)	(5.36)	(6.08)	(6.46)[b]	(7.[illegible]1)[b]
Spleen, mg	162 ± 7	136 ± 11	112 ± 7[b]	82 ± 10[b]
(% body weight)	(0.61)	(0.52)	(0.45)[b]	(0.[illegible]7)[b]
Hematology				
Fibrinogen (mg %)	226 ± 8	209 ± 7	191 ± 12[b]	119 ± 11[b]
Clinical chemistry				
SGPT, IU/l.	50 ± 7	49 ± 3	184 ± 40	648 ± 73[b]
SGOT, IU/l.	94 ± 10	85 ± 7	215 ± 38	774 ± 51[b]
BUN, mg-%	29 ± 1	28 ± 2	31 ± 2	41 ± 2[b]
Humoral immunity				
AFC/spleen × 10^5	1.11 ± 0.10	1.19 ± 0.15	0.55 ± 0.08[b]	0.35 ± 0.05[b]
AFC/10^6 cells	1018 ± 73	1133 ± 61	791 ± 61	801 ± 69
Hemagglutination ($\log_2$ titer)	8.91 ± 0.15	9.18 ± 0.14	8.32 ± 0.27	7.91 ± 0.15[b]

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.

Table 6. Effects of dibromochloromethane upon CD-1 male mice following 14 days of exposure by gavage.

Parameter	Vehicle (11)[a]	50 mg/kg (8)[a]	125 mg/kg (8)[a]	250 mg/kg (12)[a]
Body weights, g	32.3 ± 0.7	33.5 ± 1.0	31.7 ± 0.07	25.8 ± 0.8[b]
Organ weights				
Liver, mg	1851 ± 72	2148 ± 101	2376 ± 182	2007 ± 105
(% body weight)	(5.72)	(6.40)	(7.49)[b]	(7.74)[b]
Spleen, mg	162 ± 7	154 ± 10	145 ± 8	96 ± 8[b]
(% body weight)	(0.50)	(0.46)	(0.46)	(0.37)[b]
Hematology				
Fibrinogen (mg %)	309 ± 10	310 ± 5	296 ± 8	251 ± 15[b]
Clinical chemistry				
Glucose, mg-%	274 ± 14	289 ± 14	252 ± 12	151 ± 8[b]
SGPT, IU/l.	48.6 ± 5.8	68.4 ± 7.9	83.5 ± 11.1	486.1 ± 61.9[b]
SGOT, IU/l.	97.9 ± 10.8	101.9 ± 10.3	102.8 ± 11.1	474.6 ± 67.5[b]
Humoral immunity				
AFC/spleen × 10^5	1.50 ± 0.16	1.39 ± 0.13	1.01 ± 0.28	0.74 ± 0.16[b]
AFC/10^6 cells	1611 ± 115	1484 ± 165	1114 ± 191[b]	1088 ± 105[b]
Hemagglutination ($\log_2$ titer)	9.41 ± 0.08	9.57 ± 0.16	9.32 ± 0.00	8.82 ± 0.26[b]
Cell-mediated immunity				
Popliteal lymph node (Stimulation Index)	11.06 ± 0.95	8.33 ± 1.85	8.27 ± 1.12	4.30 ± 0.93[b]

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.

tion in body weight in the males at the high dose (Table 6). Liver weights were increased at 125 and 250 mg/kg/day when expressed as percent of body weight. Spleen and thymus values decreased significantly at the high dose whether the data was expressed as total milligrams or as percent of body weight. Of the hematological parameters, only fibrinogen was affected, with a decrease occurring at the high dose. Clinical chemistry alterations were increases in SGPT and SGOT and a decrease in serum glucose, all in the high dose group. Both humoral and cell-mediated immunity were affected by dibromochloromethane. The number of AFC was significantly reduced when expressed as total cells or as AFC/10^6 spleen cells. This was noted at the high dose, as was a reduction in hemagglutination titer. Cell-mediated immunity, as measured by the popliteal lymph node stimulation index, was depressed at the high dose. It should be noted that even though the changes were significant only at the 250 mg/kg/day level, the decreasing trend can be observed with the lower doses.

No significant body weight change was observed in females receiving dibromochloromethane. The major organ weight change in the females was an increase in liver size in the intermediate and high dose groups (Table 7). The ability of the liver to metabolize hexobarbital was impaired at the inter-

Table 7. Effects of dibromochloromethane upon CD-1 female mice following 14 days of exposure by gavage.

Parameter	Vehicle (12)^a	50 mg/kg (8)^a	125 mg/kg (8)^a	250 mg/kg (12)^a
Body weights, g	29.9 ± 0.5	27.1 ± 0.7	26.1 ± 0.9	26.3 ± 0.7
Organ weights				
Liver, mg	1528 ± 56	1622 ± 61	1735 ± 93	1923 ± 62^b
(% body weight)	(5.67)	(5.98)	(6.63)^b	(7.33)^b
Hematology				
Fibrinogen (mg %)	260 ± 6	259 ± 6	248 ± 10	233 ± 7^b
Clinical chemistry				
Glucose, mg-%	157 ± 4	148 ± 10	160 ± 8	105 ± 17^b
SGPT, IU/l	54 ± 6	73 ± 14	107 ± 16	449 ± 44^b
SGOT, IU/l	112 ± 8	124 ± 17	156 ± 17	526 ± 53^b
Hexobarbital sleeping time, min^c	15.1 ± 0.9	17.3 ± 1.4	27.7 ± 1.9^b	37.4 ± 2.5^b
Humoral immunity				
AFC/spleen $\times 10^5$	1.34 ± 0.11	1.00 ± 0.12	0.79 ± 0.19^b	0.45 ± 0.07^b
AFC/10^6 cells	1641 ± 85	1418 ± 80	1071 ± 195^b	1013 ± 100^b
Hemagglutination ($\log_2$ titer)	9.49 ± 0.17	9.70 ± 0.18	9.20 ± 0.30	8.82 ± 0.15^b

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.
[c] Induced by 80 mg/kg hexobarbital.

Table 8. Effects of tribromomethane upon CD-1 male mice following 14 days of exposure by gavage.

Parameter	Vehicle (10)^a	50 mg/kg (7)^a	125 mg/kg (7)^a	250 mg/kg (10)^a
Body weights, g	23.0 ± 0.9	24.5 ± 0.8	26.1 ± 0.3^b	25.8 ± 0.52^b
Organ weights				
Liver, mg	1337 ± 68	1485 ± 65	1682 ± 28^b	1779 ± 53^b
(% body weight)	(5.79)	(6.06)	(6.44)^b	(6.90)^b
Hematology				
Fibrinogen (mg %)	302 ± 24	283 ± 34	274 ± 16	218 ± 17^b
Prothrombin time, sec	7.4 ± 0.1	6.8 ± 0.2^b	7.0 ± 0.1^b	6.8 ± 0.2^b
Clinical chemistry				
Glucose, mg-%	233 ± 9	223 ± 11	204 ± 12	195 ± 8^b
SGOT, IU/l	92 ± 6	110 ± 19	80 ± 8	159 ± 33^b
BUN, mg-%	36 ± 1	36 ± 1	34 ± 1	31 ± 1^b
Humoral immunity				
AFC/spleen $\times 10^5$	6.07 ± 0.6	5.95 ± 0.6	6.69 ± 1.1	3.44 ± 0.5^b
AFC/10^6 cells	1310 ± 75	1279 ± 99	1368 ± 52	936 ± 79^b
Cell-mediated immunity				
Delayed type hypersensitivity (Stimulation Index)	3.97 ± 0.53	2.22 ± 0.26	4.11 ± 1.21	1.96 ± 0.48^b

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.

mediate and high dose as evidenced by increased hexobarbital sleeping times. The only hematological change was a slight decrease in fibrinogen at the high dose. Altered clinical chemistry parameters were increases in SGPT and SGOT and a decrease in serum glucose, all occurring at 250 mg/kg/day. A decrease in AFC/spleen and AFC/10^6 spleen cells was noted in the intermediate and high dose groups, whereas a reduction of hemagglutination titer occurred only at the high dose.

Tribromomethane. Tribromomethane caused an increase in body weight in the intermediate and high dose groups of the males (Table 8). Liver weight, expressed as total milligrams or percent of body weight, increased at the 125 and 250 mg/kg dosages. Spleen weight was unchanged. Hematological changes included a decrease in fibrinogen at the highest dose and a reduction in prothrombin time occurring at all dosages. Clinical chemistry changes all appeared at the 250 mg/kg level and included a decrease in serum glucose and BUN and an increase in SGOT. Both humoral and cellular immunity were depressed in the high dose group. The change in humoral immunity was evident whether expressed as AFC/spleen or AFC/10^6 cells.

In contrast to the males' change in body weight, females showed a significant decrease at the high dose (Table 9). An increase in liver weight was evident at the high dose regardless of the method of expression, whereas the decrease in spleen weight was significant only when expressed as total milligrams and occurred at the intermediate and high

Table 9. Effects of tribromomethane upon CD-1 female mice following 14 days of exposure by gavage.

Parameter	Vehicle (12)[a]	50 mg/kg (7)[a]	125 mg/kg (6)[a]	250 mg/kg (7)[a]
Body weights, g	22.3 ± 0.6	20.4 ± 0.5	20.6 ± 0.5	19.5 ± 0.9[b]
Organ weights				
Liver, mg	1253 ± 48	1298 ± 47	1248 ± 46	1524 ± 69[b]
(% body weight)	(5.61)	(6.37)	(6.07)	(7.[illegible])[b]
Spleen, mg	119 ± 9	99 ± 11	89 ± 6[b]	53 ± 5[b]
(% body weight)	(0.53)	(0.48)	(0.46)	(0.[illegible]7)
Clinical chemistry				
SGOT, IU/l.	80.4 ± 6.2	75.5 ± 8.6	65.8 ± 13.5	129.7 ± 17.2[b]

[a]Number of mice per group given in parentheses following each dose level.
[b]Significant at $p < 0.05$ level, Dunnett's *T*-test.

Table 10. Effects of trichloromethane upon CD-1 male mice following 90 days of exposure by gavage.

Parameter	Vehicle (11)[a]	50 mg/kg (8)[a]	125 mg/kg (7)[a]	250 mg/kg (12)[a]
Body weights, g	36.1 ± 0.9	35.5 ± 0.8	34.1 ± 1.3	36.3 ± 0.8
Organ weights				
Liver, mg	1921 ± 69	2020 ± 70	2025 ± 130	2428 ± 115[b]
(% body weight)	(5.31)	(5.69)	(5.91)	(6.[illegible])[b]
Spleen, mg	156 ± 13	139 ± 12	141 ± 12	180 ± 9
(% body weight)	(0.43)	(0.39)	(0.41)	(0.[illegible])
Clinical chemistry				
Glucose, mg-%	225 ± 11	265 ± 9	286 ± 13	318 ± 15[b]
SGPT, IU/l.	63 ± 4	58 ± 6	76 ± 6	81 ± 6
SGOT, IU/l.	90 ± 8	105 ± 12	106 ± 14	131 ± 21
Humoral immunity				
AFC/spleen × 10^5	2.27 ± 0.20	1.50 ± 0.21[b]	2.41 ± 0.27	1.34 ± 0.18[b]
AFC/10^6 cells	1723 ± 91	1371 ± 151	1620 ± 115	1081 ± 105[b]
Hemagglutination ($\log_2$ titer)	9.50 ± 0.18	9.20 ± 0.13	9.32 ± 0.22	9.16 ± 0.17
Hepatic microsomal activities				
Microsomal protein, mg/g liver	26.9 ± 0.64	24.4 ± 0.60	26.4 ± 0.69	23.5 ± 1.09[b]
Glutathione, mmole/g liver	10.0 ± 0.39	10.6 ± 0.36	10.6 ± 0.37	10.1 ± 0.48
Aniline hydroxylase, nmole/mg/min	1.86 ± 0.06	2.22 ± 0.11[b]	1.81 ± 0.02	1.52 ± 0.06[b]
Hexobarbital sleeping time (min)[c]	36.2 ± 4.0	41.8 ± 4.1	46.8 ± 4.6	47.4 ± 4.9

[a]Number of mice per group given in parentheses following each dose level.
[b]Significant at $p < 0.05$ level, Dunnett's *T*-test.
[c]Induced by 80 mg/kg hexobarbital.

doses. The only alteration in clinical chemistry values was an increase in SGOT, which occurred in the 250 mg/kg group. The immune system was not affected by tribromomethane as measured by the humoral and cell mediated responses to sRBC.

Subchronic Ninety-Day Study

CD-1 male and female mice were gavaged with trichloromethane at 50, 125 or 250 mg/kg/day for 90 days. No chemically induced deaths were attributed to the compound during treatment.

Male mice showed no significant changes in body weight due to treatment (Table 10). Liver weight was increased both as total milligrams and as percent of body weight at the high dose. Spleen weight was not affected. Clinical chemistry values were essentially normal except for serum glucose, which was elevated at the intermediate and high doses. Humoral immunity was affected at the 2[illegible]0 mg/kg dose in that AFC/spleen and AFC/10^6 cells were decreased. Total AFC/spleen also decreased at the low dose. Hemagglutination titer was not affected.

Hepatic microsomal activities were evaluated in the 90-day exposure study. Table 10 shows a suppression of microsomal protein at the high dose. There were also changes in the microsomal enzyme aniline hydroxylase, but they were not dose-related, increasing at the low dose and decreasing at the high dose.

Ninety-day trichloromethane administration did not alter body weights at any level of treatment in the female mice (Table 11). Liver weights were increased at all levels of treatment when expressed either as total milligrams or as percent of body weight. Serum glucose was increased in the females at the high dose only. The only change occurring in humoral immunity was a decrease in AFC/10[illegible] spleen cells at the low dose; not a dose-related effect. A

Table 11. Effects of trichloromethane upon CD-1 female mice following 90 days of exposure by gavage.

Parameter	Vehicle (12)[a]	50 mg/kg (7)[a]	125 mg/kg (7)[a]	250 mg/kg (12)[a]
Body weight, g	28.6 ± 0.5	29.2 ± 1.1	30.6 ± 0.5	30.8 ± 0.9
Organ weights				
Liver, mg	1293 ± 47	1606 ± 60[b]	1835 ± 42[b]	1991 ± 66[b]
(% body weight)	(4.51)	(5.54)[b]	(5.99)[b]	(6.48)
Spleen, mg	168 ± 12	158 ± 14	187 ± 25	161 ± 8
(% body weight)	(0.59)	(0.55)	(0.62)	(0.52)
Clinical chemistry				
Glucose, mg-%	232 ± 6	248 ± 15	261 ± 16	295 ± 9[b]
Humoral immunity				
AFC/spleen × 10^5	2.01 ± 0.24	1.56 ± 0.27	1.58 ± 0.14	1.76 ± 0.23
AFC/10^6 cells	1324 ± 63	984 ± 59[b]	1191 ± 79	1086 ± 79
Hemagglutination ($\log_2$ titer)	9.74 ± 0.19	9.66 ± 0.21	9.32 ± 0.22	9.66 ± 0.19
Cell-mediated immunity				
Delayed type hypersensitivity (Stimulation Index)	4.12 ± 0.56	3.14 ± 0.41	3.39 ± 0.57	2.48 ± 0.30[b]
Hepatic microsomal activities				
Microsomal protein, mg/g liver	28.0 ± 0.79	24.9 ± 0.84	22.9 ± 1.11[b]	22.3 ± 1.03[b]
Glutathione, mmole/g liver	6.91 ± 0.24	9.99 ± 0.47[b]	10.79 ± 0.50[b]	11.20 ± 0.51[b]
Aniline hydroxylase, nmole/mg/min	1.48 ± 0.04	1.41 ± 0.08	1.11 ± 0.06[b]	1.09 ± 0.06[b]
Hexobarbital sleeping time (min)[c]	19.2 ± 2.3	34.0 ± 2.9	35.0 ± 1.8[b]	46.5 ± 4.4[b]

[a] Number of mice per group given in parentheses following each dose level.
[b] Significant at $p < 0.05$ level, Dunnett's *T*-test.
[c] Induced by 80 mg/kg hexobarbital.

Table 12. Hyporesponsiveness to lethal effects of trichloromethane.[a]

Group	Females		Males	
	Survivors/*N*	% Mortality	Survivors/*N*	% Mortality
Vehicle	7/11	36	1/9	89
50 mg/kg	8/8	0	5/9	44
125 mg/kg	8/8	0	5/6	17
250 mg/kg	7/7	0	7/8	13

[a] Animals were given a single gavage of 1000 mg/kg after 90 days of exposure to the doses listed.

decrease was noted in the cell-mediated delayed type hypersensitivity assay, occurring in the high dose group.

The kidneys, livers and spleens of male and female mice gavaged with vehicle did not exhibit histopathological changes. Histologic examination of the selected organs of mice gavaged with chloroform revealed normal spleens; however, slight histopathologic changes were seen in the kidneys and livers of both males and females. The kidneys exhibited small intertubular collections of chronic inflammatory cells, mainly lymphocytes. The liver exhibited generalized hydropic degeneration of hepatocytes and occasional small focal collections of lymphocytes. Small amounts of extravasated bile were occasionally noticed in the females. Most of this extravasated material was present within sinusoidal Kupffer cells.

The females displayed a slight decrease in microsomal protein at the intermediate and high doses (Table 11). Aniline hydroxylase activity also decreased at these doses. Glutathione increased at all three levels of treatment, a change not observed in the males.

Tolerance Study

As shown in Table 12, a degree of protection to the lethal effects of trichloromethane was induced by subchronic 90-day exposure. A single 1000 mg/kg dose was lethal to 36% of the females who had received vehicle for 90 days but none of the treated mice died. Mortality of vehicle-treated males was 89%, while in the trichloromethane-treated groups, 13–44% died.

Discussion

A result of industrialization and urban development has been an increased need for a monitoring system to assess environmental contamination pertaining to air, food, and water supplies. Since it is impractical, if not impossible, to completely eliminate many low-level contaminants, it is imperative that we understand the effects of exposure to these contaminants. Our efforts to insure potable water supplies through chemical treatment have contributed to the ever-increasing number of compounds to which we are exposed. The purpose of this study was to examine the effects of low-level exposure to selected halomethanes which are found in finished water supplies as a result of chlorination or industrial wastes. In addition to standard toxicological evaluation, the main intent was to investigate the effects of these chemicals upon the immune system. The use of the random-bred CD-1 mouse was a compromise between the random-bred rat, which is used for conventional toxicity testing, and the inbred mouse, which is most suitable for studies involving immune system function. Gavage was selected as the route of administration to control the amount of chemical to which the animals were exposed.

The major effect seen in subchronic 14-day administration of the compounds in regard to organ weights was an increase in liver weight. This was evident for all compounds and usually occurred in the intermediate and high dose groups of both sexes. This effect seemed to carry over into the 90-day study as was more evident in the female groups, in which liver weight was increased at all doses, whereas a significant change was only seen at the high dose in the males. It should be noted that in a number of instances the spleen weight decreased. This is of importance since the spleen is intimately involved with immune system function.

Hematological studies did not reveal any major changes in the cellular components of this system. However, a decrease in fibrinogen levels was noted with dibromochloromethane, tribromomethane and bromodichloromethane administration. This may reflect decreased capacity of the liver to synthesize the protein.

Alterations which occurred in the clinical chemistry parameters were indicative of hepatic disturbances. SGPT or SGOT were increased in both sexes with all compounds administered in the 14-day studies. In the 90-day study, this effect was not seen, perhaps indicative of recovery or tolerance to the compound used in the long-term experiment. The elevation in serum glucose level in the subchronic 90-day exposure may also reflect alteration in the ability of the liver to control glucose levels.

Although cell-mediated immunity was depressed in a few instances, these changes occurred only with the highest dose. Suppression of the humoral immune system was observed with all the chemicals under investigation in both sexes, the only exception being the females receiving tribromomethane for 14 days. Furthermore, a dose response was evident in almost all instances and, even when the decreases were not statistically significant, a definite trend toward reduction could be noticed. Thus it appears that the humoral immune system may be an indicator of halomethane toxicity. Further studies involving functional aspects of immunity are necessary to strengthen this observation.

The observation that subchronic 90-day exposure to trichloromethane did not exacerbate the changes seen to occur as a result of 14-day administration of the compound and, in fact, were less severe, points out the need for short-term studies to reveal effects which although transient, may have serious consequences under given circumstances, e.g. exposure to pathogens while the immune system is depressed. This is supported by the tolerance experiment, which revealed compensatory mechanisms being activated during subchronic 90-day exposure.

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Environmental Health Perspectives
Vol. 46, pp. 127-136, 1982

Behavioral Toxicity of Trihalomethane Contaminants of Drinking Water in Mice

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The behavioral toxicity of trichloromethane (TCM), dichlorobromomethane (DCBM), dibromochloromethane (DBCM) and tribromomethane (TBM) was evaluated following oral administration in mice. A variety of dosage regimens and behavioral measures were used. Studies included acute dose effect, 14- and 90-day treatments at 300 and 3000 times the estimated average human daily intake of contaminated drinking water, 30 days of 100 mg/kg/day, and 60 days of 100 and 400 mg/kg/day. In addition, TCM was tested for the production of taste aversions with 10-day administration and for behavioral teratology in offspring following extensive perinatal exposure. The ED_{50} for acute effects on a screen test of motor performance was about 500 mg/kg for all four trihalomethanes. The 14-day treatments had no effect on swimming behavior and the 90-day treatments had no effect on bar clinging, a test of motor coordination, and a measure of exploratory behavior. None of the compounds produced effects on passive-avoidance learning following 100 mg/kg/day for 30 days. TCM, DBCM and TBM elicited clear effects at both 100 and 400 mg/kg/day on operant behavior when administered for 60 days. DBCM elicited clear effects at 400 mg/kg/day. These effects on operant behavior were seen following the first dose and tolerance tended to develop. Thus, there was no evidence from these studies for a progressive neurotoxicity from trihalomethanes in adult mice. A behavioral teratology study was also conducted with TCM. Both parents were treated with 31.1 mg/kg/day TCM, and treatment of the dam continued throughout gestation and lactation. No clear evidence for behavioral effects in the offspring were observed. The most sensitive measure for the effects of TCM was the taste aversion paradigm in which saccharin aversions were produced after a single treatment of 30 mg/kg.

Trihalomethanes are present in finished drinking water in relatively large amounts due to their formation during water disinfection by chlorination (*1–3*). As part of a larger project to assess the toxicity of drinking water contaminants in mice, a number of studies of the behavioral effects of four trihalomethanes were conducted. This paper describes our early approach to behavioral toxicity testing and reviews the results.

Several considerations led to the design of the experiments to be reported here. The mouse was chosen as the experimental subject largely due to its planned use by other components of the overall project. The mouse is not commonly used in behavioral toxicology hence most of the tests were developed or adapted specifically for this project. Concern with drinking water exposure dictated that the studies use an oral route of administration and that the studies include long term, daily, low dose exposures. Since the behavioral toxicity of these types of exposures to trihalomethanes had not been evaluated, there was no basis for predicting which behavioral functions would be most likely affected by these contaminants. For this reason, a battery of behavioral tests were used and many different exposure conditions were evaluated. Finally, some of the dosage levels were chosen based upon the highest levels reported in finished water supplies at the time this research was initiated (*1*). Most of the doses for long-term exposure studies were chosen to represent 300 and 3000 times the estimated average daily human intake (ADI) assuming 2 liters per day intake of these highly contaminated supplies.

Methods

Subjects

With the exception of the behavioral teratology and taste aversion experiments, all studies were carried out with adult male ICR mice (Flow Laboratories, Dublin, Va.). They arrived in the weight range of 19–24 g and were initially housed in groups of eight to ten in standard laboratory mouse cages equipped with automatic watering using tap water. They were allowed at least one week of adaptation before any experimentation was carried out. With the exception of food deprivation for operant studies, mice were allowed continuous access to Ralston-Purina Rodent Laboratory Chow no. 5001. No special precautions were taken to use contaminant-free water since we were interested in studying large doses of selected contaminants administered by gavage. Consumption of Richmond tap water was considered to be a more natural background against which the effects of the trihalomethanes could be studied.

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After the adaptation period, subjects were assigned randomly to groups. For most studies this resulted in cages of six to eight mice receiving identical treatments. For taste aversion and operant studies the subjects were individually housed. For the teratology studies both female and male ICR mice were used, and the behavioral studies were carried out on the offspring. For the taste aversion study, adult male mice of the CD-1 strain (Charles River, Wilmington, Mass.) were used.

Behavioral Tests

A number of different behavioral tests were used to assess the effects of both acute and repeated trihalomethane administration. With the exception of the operant studies, all testing was carried out by an experimenter blind as to the dosing conditions. The following is a description of the behavioral tests.

Screen Test. This is a simple test of motor performance described originally by Coughenour et al. (*4*) and is similar in some respects to the more common rotorod. Mice were placed individually on top of a horizontal wire mesh screen 13 × 13 cm square placed 31 cm above the table top. Six such screens were mounted in a row on a metal rod which, when rotated, turned the screens upside down. The number of mice which climbed to the top of the screen by 15, 30 and 60 sec was counted. This measure was used to study the acute effects for each of the trihalomethanes as well as the motor performance of mice receiving repeated doses. For the latter, treatment groups were compared to control groups by chi-square analysis. Two tests were conducted 30 min apart beginning 24 hr after the last dose, and the results at 15, 30 and 60 sec for each test were analyzed separately.

Cling Test. Mice were tested for their ability to cling to a small diameter bar suspended horizontally 29 cm above a table surface. Latency to fall was recorded up to a maximum of 60 sec. Treatment and control groups were compared by one-way analysis of variance.

Hole-Board. The hole-board has been used by a number of investigators (*5,6*) to assess exploratory activity. The apparatus consisted of a 43-cm square plastic box with a false floor positioned 17 cm above the bottom. The bottom was divided into four sections, and two of the sections contained a complex object consisting of hardware (nuts, bolts and wires) attached to a wooden block, and two were empty. A 3-cm diameter hole in the false floor was situated directly above each of the four compartments. Mice were placed individually on the false floor and the number of head dips into holes above compartments with or without objects were recorded separately. Head dips were scored by an observer when the subjects' nose and eyes were inserted into the hole. The apparatus was wiped clean after the testing of each subject. The test period was 3 min and subjects were tested 24 hr after the last of repeated doses of the trihalomethanes. Treatment and control groups were compared by an unweighted means two-way analysis of variance with repeated measures on one factor (groups × objects present or absent).

Swimming Endurance. Swimming behavior was assessed in a 30 × 30 cm cylindrical tank containing water at 15°C. Animals were made heavier by taping a length of lead wire equal to 10% of the subjects' body weight onto their back. The weight was placed in a way not to interfere with leg movement. A line was drawn 2 cm below the water level and the latency to sink below the line was recorded. The animals were then immediately removed from the tank. No subjects drowned in carrying out these studies. Animals were tested individually 24 hr after the last dose of repeated dosing. Results for treatment and control groups were compared by one-way analysis of variance.

Passive-Avoidance Learning. One trial passive shock avoidance was assessed following repeated contaminant exposure. A trapezoidal-shaped box was divided into a clear "safe" compartment and a dark "shock" compartment by a guillotine door. On a training trial a subject was placed in the safe compartment and the latency to enter the dark compartment was recorded. When the subject entered the dark compartment the door was lowered and the subject was given 5 sec of unavoidable 40-V ac shock via grid plates comprising the floor of the shock compartment. One hour and 24 hr later, test trials were conducted. They were identical to the training trial except shock was omitted. Training trials were conducted 24 hr following the last repeated dose of the trihalomethanes. The latencies for treatment and vehicle control groups were compared by two-way analysis of variance with repeated measures on one factor (groups × test period).

Operant Behavior. Studies of the effects of repeated administration of trihalomethanes on schedule-controlled performance were conducted in operant chambers designed for this study. The apparatus and general procedures for care and feeding of subjects, lever press training, and experimental control and data collection have been described previously (*7*). Briefly, individually housed mice were given 2 hr per day restricted access to food following daily 30-min experimental sessions. They were trained to lever press for dipper presentations of sweetened milk on a differential reinforcement of low rate 10 sec (DRL 10) schedule, i.e., only interresponse times of 10 sec or greater were reinforced. Subjects were trained over a period of about two months until response rates were stable. Subjects were then habituated to daily vehicle gavage (5 ml/kg Emulphor:water 1:8) given 30 min prior to the onset of the session. At this point daily treatment was initiated. The subjects in each treatment group (N = 6 to 13) received a daily intubation 30 min prior to each session, seven days per week. On occasional days when sessions were not conducted, treatments were continued as usual. Daily treatments were given for 60 days or until the subjects died, which commonly occurred in high dose groups. After 60 days the treatments were discontinued and the subjects were tested for an additional 3 days following vehicle gavage. The primary measures of behavior were response rates and reinforcement rates. These measures for the last pretreatment session and the first treatment session were compared using a t-test of differences.

Neurobehavioral Development. The methods and results of behavioral teratology evaluation of trichloromethane (TCM) have been fully reported elsewhere (*8*). The offspring of mice treated throughout gestation and lactation were assessed for possible behavioral effects. A battery of tests of neurobehavioral development, which included measures of the righting reflex, the forelimb placing response, forepaw grasping, the rooting reflex, cliff drop aversion, auditory startle and bar-holding ability were assessed daily from birth through day 15. The screen test as described above was carried out on day 17 and passive avoidance learning on days 22 and 23.

Taste Aversion. Taste aversions produced by TCM in adult mice have been fully described elsewhere (*9*). Individually housed mice were gradually adapted to 30 min per day access to water. After fluid consumption stabilized they were given access to a solution of 0.3% sodium saccharin during the 30-min drinking period. Immediately following the saccharin drinking session the subjects were gavaged with a dose of TCM or vehicle (N = 10 per group). For 10 subsequent days, the subjects were given a two bottle preference test between saccharin and water and following each test the appropriate treatment was administered. Illness-inducing effects are seen as a conditioned aversion to the saccharin solution with which the treatment has been paired. Data are expressed as percent saccharin consumed during the preference tests.

Treatments

The four trihalomethanes with their code, drinking water concentration on which some of the doses were chosen, and estimated average daily human intake (ADI) are presented in Table 1. Both acute effects and the effects of varying durations of repeated administration were assessed. Doses for the 14 and 90-day and behavioral teratology study were chosen to represent 300 and 3000 times the estimated ADI. Higher doses were chosen for other studies in an attempt to determine minimally active dosage regimens.

The trihalomethanes were administered by gavage in suspension using a vehicle of 1:8 Emulphor:water. Emulphor (EL-620, GAF Corp., NY, NY) is a polyoxyethylated oil nonionic surfactant useful for suspending water-insoluble agents for administration to laboratory animals (*10*). We have found that large volume gavage can affect mouse behavior when administered shortly before testing. Thus, for studies in which testing was carried out within a

Table 1. Trihalomethanes studied for behavioral toxicity.

Compound	Abbreviation	Highest concentration, μg/l.[a]	Average daily intake μg/kg/day[b]	Lowest doses studied, mg/kg/day[c]
Trichloromethane	TCM	311	10.4	3.1 and 31.1
Dichlorobromomethane	DCBM	116	3.9	1.2 and 11.6
Dibromochloromethane	DBCM	100	3.3	1.0 and 10.0
Tribromomethane	TBM	92	3.1	0.9 and 9.2

[a] Highest concentration reported in finished drinking water (*1*).

[b] Estimated average human daily intake based upon highest concentrations reported in drinking water.

[c] Doses used for 14 and 90 day studies.

few hours of dosing (operant behavior, acute dose-effect) a volume of 5 ml/kg was used. For long-term studies the injection volume was 10 ml/kg. Fresh suspensions were prepared daily. For all repeated dosing experiments the subjects were gavaged daily, 7 days per week, for the duration of the exposure. For some of the studies, a noninjected control group was included.

Acute Effects. Acute dose-effect curves were obtained for all four trihalomethanes on the screen test. Tests were conducted 30, 60 and 90 min after intubation. Five or six doses of each compound were tested ($N = 6$ per dose). ED_{50} values were estimated by a computer approximation (*11*) to the method of Bliss (*12*).

The conditioned taste aversion evaluation could also be considered a measure of the acute effects of TCM since the effects appeared after the first of 10 doses. Oral doses of 3, 10 and 30 mg/kg as well as a vehicle control were assessed.

14-Day Administration. Two dose groups of each trihalomethane (300 and 3000 times the estimated ADI; Table 1), as well as vehicle and noninjected control groups ($N = 8$ per group), were treated for 14 days. Swimming behavior was assessed 24 hr following the last treatment.

90-Day Administration. Two doses of each trihalomethane (300 and 3000 times the estimated ADI; Table 1), as well as separate concurrently treated vehicle and noninjected control groups, were exposed for 90 days. Generally eight subjects were included in each group; however, the vehicle control group for DBCM contained six subjects, the high dose DCBM group contained seven subjects, and both DBCM groups contained 11 subjects. Behavioral testing was carried out over 2 days beginning 24 hr after the last treatment. Subjects were evaluated on the screen test, the hole board and the cling test.

30-Day Administration of 100 mg/kg. The four trihalomethanes were administered at 100 mg/kg/day for 30 days. A concurrent vehicle control group was also tested with each compound. Both treatment and control groups consisted of 16 subjects. Passive-avoidance learning was evaluated beginning 24 hr following the final treatment.

60-Day Administration of 100 and 400 mg/kg. Groups of 6-13 mice trained on a DRL 10 sec schedule of milk presentation were given oral treatments 30 min prior to daily behavioral testing for 60 days. Two dose groups of each trihalomethane (100 and 400 mg/kg) were tested as well as one vehicle control group.

Perinatal Administration. Male and female mice were gavaged with vehicle or 31.1 mg/kg/day TCM for 21 days prior to mating, throughout mating (21 days or until a vaginal plug was detected), and the dam was continued with daily gavage throughout gestation and lactation. The pups were also gavaged with the same dose beginning on day 7. Five TCM and five control litters were evaluated for behavioral teratology. Additional details on the methods for this study can be read elsewhere (*8*).

Results

In general the trihalomethanes were without effects in most of the behavioral tests under most exposure conditions. TCM was investigated most thoroughly including behavioral teratology evaluation as well as for ability to produce a taste aversion. It was active at 100 and 400 mg/kg/day in disrupting operant behavior; however, tolerance developed to this effect. A single oral dose of 30 mg/kg resulted in a clear taste aversion and tolerance did not develop to this effect over 10 days administration. TCM did not result in behavioral teratological effects at 31.1 mg/kg/day, nor was it active at 3.1 or 31.1 mg/kg/day for 14 days on swimming behavior or for 90 days on three other behavioral tests.

The other trihalomethanes were not tested for ability to produce taste aversions or for behavioral teratology. They were all active at very high doses of 100 and/or 400 mg/kg on operant behavior, but were inactive at 100 mg/kg/day for 30 days on avoidance learning. They were also inactive at doses of 300 and 3000 times the ADI for 14 days on swimming behavior and were generally inactive when given for 90 days on three other tests. Occasional significant differences between treatment and control groups were observed; but caution should be used in interpreting these since a pattern of effects did not emerge nor were greater effects seen with higher doses, and many separate statistical analyses were performed.

Since many of the results were negative, all of the data for these studies will not be presented. Rather, selected data will be shown to illustrate the conclusions stated above.

Acute Dose Effect

The acute effects of the trihalomethanes were evaluated on the screen test at 30, 60 and 90 min after oral administration. Effects were seen by 30 min with little evidence of much change by 90 min. Table 2 presents the ED_{50} values for each of the trihalomethanes at their most active time point. All four compounds were roughly equipotent. The effects observed at high doses were ataxia and incoordination and, at the highest doses, anesthesia.

Table 2. Potency estimates of acute oral trihalomethane administration on the screen test in mice.

Compound	ED_{50}, mg/kg[a]	95% confidence limits (mg/kg)	Slope[b]
TCM	484	243 – 965	–2.6
DCBM	524	273 – 1007	–2.9
DBCM	454	262 – 788	–2.0
TBM	431	238 – 788	–2.6

[a] Dose estimated to affect 50% of the subjects at time of peak effect. Calculated by the probit method of Baird and Balster (11).

[b] In log dose probit units.

Repeated Administration

14-Day Administration. Groups of subjects treated with two doses of each trihalomethane were tested for swimming endurance. The mean latency to sink for each treatment group is provided in Table 3. Analysis of variance revealed an overall significant effect of treatments [$F(9,70) = 2.33$, $p < 0.05$]; however, using Dunnett's test to compare each group to the noninjected or vehicle control revealed no one group to be significantly affected. The largest effects were increases in endurance by the low dose TCM group and the high dose TBM group. This increased endurance by the high dose TBM group further suggests that the nonsignificant decrease in endurance in the low dose TBM group was not a reliable treatment effect.

90-Day Administration. The same doses that were used in the 14-day study were used for a 90-day subchronic study. Three behavioral measures, the cling test, screen test and hole board, were used. Both doses of each trihalomethane were tested simultaneously with vehicle and noninjected controls. For the cling test and the screen test, the data for the control groups for each trihalomethane were pooled to provide a better estimate of control behavior. This was done primarily because of the relatively large within-group variability in the case of the cling test and the poor power for detecting effects for the screen test due to the nominal form of the data. This pooling resulted in 27 vehicle control subjects and 28 noninjected controls. One-way analysis of variance on the latency to fall in the cling test revealed no significant F-values in comparing treatment to the pooled control groups. The results of the chi-square tests comparing the effects of the various treatments to the pooled control data (vehicle + noninjected) for the screen test are shown in Table 4. Forty-eight separate analyses were performed. There were no effects of either dose of TCM at any of the time points. Four of the 12 analyses for DCBM revealed significant differences. In all four cases, the differences were observed in the low dose group but not in the high dose group. Two of the 12 analyses for DBCM reached statistical significance. As with DCBM, effects on the measure were observed only with the low dose, and DBCM had no effects on the other behavioral measures at this dose. TBM had no significant effect at either dose at any of the time points measured.

The effects of the 90-day treatments on the hole-board test are presented in Table 5. Two-way analyses of variance were performed on these results for each study. No significant effects of treatments nor treatments by objects interaction was observed in any of the studies. In the DBCM and TBM study the subjects explored holes with objects significantly more frequently than holes with no objects, but the object variable was not significant in either the TCM or DCBM study.

30-Day Administration of 100 mg/kg. Figure 1 presents the results of 30-day administration of the trihalomethanes on latency to enter the dark compartment in the passive avoidance learning test. On the training trial prior to shock delivery, subjects rapidly moved from the light to the dark compart-

Table 3. Effects of 14-day oral administration of trihalomethanes on swimming endurance.

Treatment group[a]	Trihalomethane dose, mg/kg/day	Mean latency to sink, sec	SEM
Noninjected control	–	79.7	19.8
Vehicle control	–	75.5	16.2
TCM	3.1	114.7	10.2
TCM	31.1	89.6	16.9
DCBM	1.2	92.2	15.5
DCBM	11.6	80.5	13.6
DBCM	1.0	65.6	9.2
DBCM	10.0	68.9	6.9
TBM	0.9	49.1	5.1
TBM	9.7	119.4	19.1

[a] $N = 8$ mice per group.

Table 4. Effects of 90-day oral administration of trihalomethanes on the screen test.

Treatment	Trihalomethane dose, mg/kg/day	N	Trial 1[a]			Trial 2[a]		
			15 sec	30 sec	60 sec	15 sec	30 sec	60 sec
TCM	3.1	10	NE	NE	NE	NE	NE	NE
TCM	31.1	11	NE	NE	NE	NE	NE	NE
DCBM	1.2	8	NE	S	S	S	S	NE
DCBM	11.6	7	NE	NE	NE	NE	NE	NE
DBCM	1.0	11	NE	S	NE	S	NE	NE
DBCM	10.0	11	NE	NE	NE	NE	NE	NE
TBM	0.9	8	NE	NE	NE	NE	NE	NE
TBM	9.2	8	NE	NE	NE	NE	NE	NE

[a] Effects at stated times sec. during trial. Trials 1 and 2 were separated by 30 min. NE = no effect; S = significantly worse than pooled control subjects by chi-square, $p < 0.05$.

Table 5. Effects of 90-day oral administration of trihalomethanes on the hole-board test.

Treatment group[a]	N	Object present		No object	
		Mean head dips	SEM	Mean head dips	SEM
I. TCM Study					
Noninjected	8	16.5	3.4	15.4	1.8
Vehicle	8	19.0	2.9	19.9	2.7
TCM, 3.1	8	21.7	3.1	21.1	3.8
TCM, 31.1	8	19.0	2.6	15.4	1.9
II. DCBM Study					
Noninjected	8	15.5	2.8	17.2	1.5
Vehicle	6	15.0	2.2	14.7	2.7
TCM, 1.2	8	15.9	1.8	15.4	1.8
TCM, 11.6	7	20.0	1.4	18.7	2.5
III. DBCM Study					
Noninjected[b]	8	15.0	1.9	13.8	3.0
Vehicle[b]	8	19.4	3.2	13.5	2.1
TCM, 1.0	11	13.0	1.9	14.4	2.6
TCM, 10.0	11	16.4	3.0	15.0	1.9
IV. TBM Study					
Noninjected[b]	8	15.0	1.9	13.8	3.0
Vehicle[b]	8	19.4	3.2	13.5	2.1
TBM, 0.9	8	19.1	2.4	16.2	1.7
TBM, 9.2	8	14.2	1.6	12.1	1.2

[a] Dose in mg/kg/day.

[b] The DBCM and TBM studies were conducted concurrently, thus the same control groups were used.

ment with an average latency of less than 10 sec. On the two retention trials 1 and 24 hr later, the latency was markedly increased indicating that the subjects learned the avoidance. Two-way analyses of variance failed to reveal any significant treatment or treatment by trial interaction. In all four studies there was a highly significant test period effect, i.e., the latencies increased from the training to the post-shock trials. Thus, 100 mg/kg/day for 30 days of each of these trihalomethanes had no effect on passive-avoidance learning or initial step-through latency on this test.

60-Day Administration of 100 and 400 mg/kg. Figure 2 shows the mean daily response rates for the vehicle control group throughout the 60 days of treatment plus the three pre- and post-treatment days. Seven subjects began this regimen. One died from a faulty injection on day 5; thus six subjects completed the study. As can be seen in Figure 2, response rates remained quite stable over the course of the study and the variability decreased as all of the subjects' performance in the task improved. Rates of reinforcement (not shown) rose steadily from 2.71 reinforcements/min on day 1 to 3.3 reinforcements/min on day 60.

Figure 3 presents the effects of 400 mg/kg/day TCM on response rates. These results were typical of the effects of active doses of the other trihalomethanes as well. Thirteen subjects began this treatment; however, six died by day 25, thus only

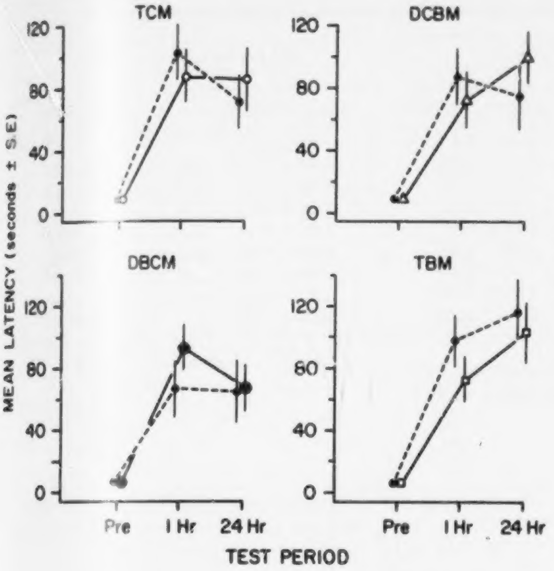

FIGURE 1. Effects of 100 mg/kg/day of (————) trichloromethane (TCM), dichlorobromethane (DCBM), dibromochloromethane (DBCM) and tribromomethane (TBM) or (---) vehicle following 30 days of oral administration on passive-avoidance learning in mice.

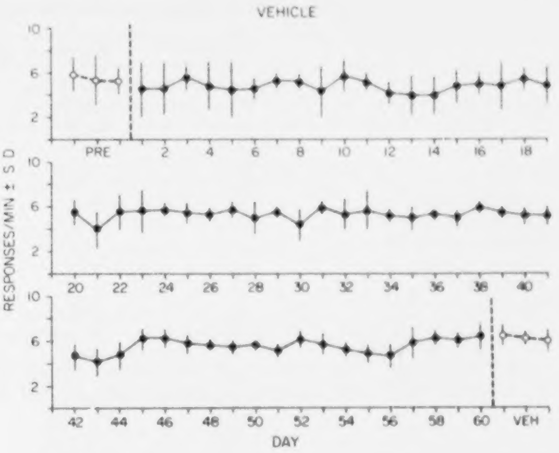

FIGURE 2. Effects of 60 days of an Emulphor:water vehicle on response rates of mice trained on a differential reinforcement of low rate 10 sec schedule of milk presentation. The three open circles prior to and following the 60 days also represent effects of vehicle administration. Treatments were administered by gavage 30 min prior to 30-min experimental sessions.

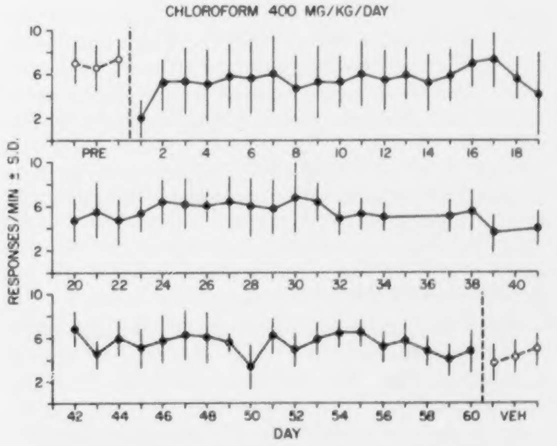

FIGURE 3. Effects of 60 days of 400 mg/kg/day trichloromethane on response rates of mice trained on a differential reinforcement of low rate 10 sec schedule of milk presentation. Details the same as in Figure 2.

seven completed this treatment regimen. Animals commonly died during these high dose regimens. Typically their schedule-controlled behavior was unchanged until a few days before they died. It can be seen from the figure that there was an initial large effect of 400 mg/kg given 30 min prior to the session. Response rates and reinforcement rates (not shown) were both substantially decreased. However, rather than a progressive deterioration of performance over the 60-day regimen, some tolerance developed to the initial effects. Variability also decreased over the 60 days although never to the extent seen in the control subjects (Fig. 2). There was no significant effect on the first day following discontinuation of TCM treatment.

This general pattern of effects on the first day of treatment with partial tolerance development was seen with all active doses of the trihalomethanes. Table 6 compares response rates and reinforcement rates on the last pretreatment day and the first treatment day in all nine groups. Significant decreases in response rate, usually accompanied by significant changes in reinforcement rates, were seen with all trihalomethane groups except the 100 mg/kg DBCM group.

Therefore, operant behavior was affected by 100 mg/kg/day of TCM, DCBM and TBM and by 400 mg/kg/day of all four trihalomethanes. Greatest effects were observed early in the regimen with no evidence for progressive behavioral deterioration; in fact, partial tolerance occurred to the effects of these contaminants.

Table 6. Effects of the first day of oral trihalomethane administration on schedule-controlled behavior.

		Responses per minute		Reinforcements per minute	
Treatment group[a]	*N*	Last pretreatment day (mean ± SEM)	First treatment day (mean ± SEM)	Last pretreatment day (mean ± SEM)	First treatment day (mean ± SEM)
Vehicle	7	4.89 ± 0.91	4.89 ± 0.95	2.93 ± 0.57	2.71 ± 1.07
TCM, 100	12	8.60 ± 0.51	6.38 ± 0.53[b]	2.06 ± 0.70	2.67 ± 0.79[b]
TCM, 400	13	7.40 ± 0.46	2.05 ± 0.53[b]	2.58 ± 0.28	1.23 ± 0.29[b]
DCBM, 100	8	7.20 ± 0.61	4.97 ± 0.76[c]	2.55 ± 0.16	2.54 ± 0.14
DCBM, 400	11	6.84 ± 0.35	2.80 ± 0.56[b]	2.99 ± 0.13	1.39 ± 0.23[b]
DBCM, 100	7	5.85 ± 1.01	5.29 ± 1.12	2.93 ± 0.34	2.50 ± 0.28
DBCM, 400	12	6.94 ± 0.61	1.74 ± 0.58[b]	2.98 ± 0.21	0.96 ± 0.26[b]
TBM, 100	8	5.95 ± 0.14	4.97 ± 0.42[c]	3.22 ± 0.15	3.23 ± 0.10
TBM, 400	6	5.95 ± 0.27	2.86 ± 1.28[c]	3.23 ± 0.23	1.62 ± 0.75[c]

[a] Dose in mg/kg/day.

[b] $p < 0.01$, paired *t*-test between last pretreatment day and first treatment day.

[c] $p < 0.05$, paired *t*-test between last pretreatment day and first treatment day.

Perinatal Administration

Only the effects of perinatal administration of TCM were evaluated. These results have been reported elsewhere (*8*). Repeated dosing of the parents through gestation and lactation and the pups from 7 days of age until weaning with 31.1 mg/kg/day had no consistent effect on neurobehavioral development nor on motor performance or passive avoidance learning. The only significant difference between the TCM and vehicle groups was in forelimb placement on days 5 and 7. Since many separate analyses were performed and no differences were seen in other measures of development of function of the extremities (forepaw grasping, bar holding), we did not place much confidence in the reliability of this TCM effect. Thus, there is little evidence of behavioral teratological effects of TCM at 31.1 mg/kg/day.

Taste Aversion

Taste aversions produced by TCM have been reported elsewhere (*9*). Oral doses of 3 and 10 mg/kg/day were without effect after the first day and an aversion to the saccharin did not appear with 10 days of treatment. A significant saccharin aversion was produced after a single pairing with 30 mg/kg TCM and over 10 days of repeated pairings the aversion increased to the point that almost no saccharin was consumed during the two-bottle choice test.

Discussion

A summary of the results of behavioral toxicity evaluation of trihalomethanes is presented in Table 7. TCM has been evaluated most extensively. Acutely, TCM is not very active on most behavioral tests. Its ED_{50} on a simple test of motor performance probably measuring the CNS depressant properties of high doses was 484 mg/kg. This dose is about one-half the LD_{50} obtained under similar conditions (*13*). On the more sensitive measures of schedule-controlled behavior, significant effects were seen at 100 mg/kg. These effects occurred with the first dose and, rather than progressive deterioration of behavior with repeated doses, there was some evidence for tolerance development. Since doses lower than 100 mg/kg were not tested with operant behavior, the minimal active dose cannot be determined, but based upon the magnitude of the effects seen we would not expect doses much lower than 100 mg/kg to have had an effect. The most sensitive measures of the acute effects of TCM was the taste aversion paradigm (*9*). Oral doses of 3 and 10 mg/kg were inactive but 30 mg/kg produced a clear effect. Although taste aversions are a behavioral measure, this does not mean that the toxic effect producing this aversion is mediated by the central nervous system. Taste aversions can be produced by many types of illness-causing agents (*14*). We do not believe that this effect of 30 mg/kg TCM was due to a localized gastric irritation, however, since an even lower dose could produce the aversion by the IP route.

The remainder of the behavioral evaluation of TCM was designed explicitly not to measure acute effects since testing was not carried out until 24 hr following the last treatment. In these studies we were looking for evidence of relatively permanent behavioral changes. A variety of measures and dosage regimens were used, but even up to 100 mg/kg/day for 30 days there was no evidence for behavioral toxicity. The measures used (screen test, cling test, hole-board, swimming and passive-

Table 7. Summary of behavioral toxicity evaluation of trihalomethanes.

	Effect[a]			
	TCM	DCBM	DBCM	TBM
Acute ED_{50} mg/kg	484	524	454	431
14-day, swimming				
300 times ADI/day	No	No	No	No
3000 times ADI/day	No	No	No	No
90-day, cling, screen and hole-board				
300 times ADI/day	No	No[b]	No[c]	No
3000 times ADI/day	No	No	No	No
30-day 100 mg/kg, learning	No	No	No	No
60-day, operant behavior				
100 mg/kg/day	Yes	Yes	No	Yes
400 mg/kg/day	Yes	Yes	Yes	Yes
Perinatal administration				
31.1 mg/kg/day	No	NT	NT	NT
Taste aversion				
3 mg/kg/day	No	NT	NT	NT
10 mg/kg/day	No	NT	NT	NT
30 mg/kg/day	Yes	NT	NT	NT

[a] No = no effect; yes = significant effect; NT = not tested.
[b] Four of 12 screen tests were significant, but no clear pattern suggesting behavioral toxicity.
[c] Two of 12 screen tests were significant, but no clear patterns suggesting behavioral toxicity.

avoidance learning) were not selected to be particularly sensitive, but rather as initial screening tools. Had effects at these high doses been found, lower doses would have been tested in more sensitive measures (e.g., operant behavior). In addition, effects were not seen as a result of perinatal administration of 31.1 mg/kg/day. This study can be considered an initial screening of the behavioral toxicity associated with long-term oral TCM exposure. There is no evidence from these results with doses 300 and 3000 times or more the ADI of TCM from contaminated drinking water that progressive neurobehavioral toxicity is likely to be a problem.

The other trihalomethanes were not evaluated quite as extensively as TCM, but what data there is suggests that they, too, are not very active behaviorally (Table 7). The ED_{50} values of DCBM, DBCM and TBM were comparable to those for TCM. With the exception of DBCM they all had effects at 100 mg/kg on operant behavior. All the trihalomethanes had pronounced effects on operant behavior at 400 mg/kg/day. Like these effects of TCM, effects on operant behavior were greater early in the 60-day treatment regimen; thus they were not progressive. Fourteen and 90-day studies of 300 and 3000 times the ADI of DCBM, DBCM and TBM were without consistent behavioral effects. Occasional significant differences were observed between treatment and control groups but these should be interpreted with caution. For example, 90-day administration of the low dose of DCBM (1.2 mg/kg/day) had a significant effect on the screen test of motor coordination on 4 of the 12 analyses performed. In assessing the implications of this, it should be kept in mind that: (1) with a large number of statistical analyses performed some will be significant by chance; (2) these four differences are not independent observations, since once a subject has fallen from the screen by 30 sec (as six did at the low dose on trial 1), they will also be counted as fallen at 60 sec; (3) the effects were only seen with the low dose; (4) no effect of DCBM was seen in the cling test or hole-board tested in these same subjects. Nevertheless, it is possible that DCBM may have some neurobehavioral effects that were not dose-dependent and which may need to be evaluated by further research. However, even 30-day treatment with 100 mg/kg/day of any of the trihalomethanes had no effects on any of the measures obtained from a passive avoidance learning task. We have not evaluated DCBM, DBCM or TBM for behavioral teratology and they have not been tested in the taste aversion paradigm. The latter would be most interesting to do since it was most sensitive to the effects of TCM.

The evaluation of the behavioral toxicity of trihalomethanes represented our first efforts in the assessment of the safety of drinking water contaminants. As a result of this experience, we have made a number of changes in the behavioral battery as well as in the choice of dosage regimens (*15,16*). Subsequent studies have not based the choice of doses on ADI's estimated from reports of levels of drinking water contamination. Rather, an attempt is now made to determine maximal no-effect levels. This is done by choosing doses for repeated exposure based upon the acute behavioral and lethal effects. Doses for 14-day studies are chosen as 1/10 and 1/100 of the LD_{50} for the chemical. If no adverse behavioral effects are observed in the subacute study, then the subchronic study is executed at the same doses. In addition, where feasible, the subchronic studies are carried out by exposing subjects via their drinking water rather than by daily gavage. If this approach to dosage choices had been used with the trihalomethanes, then the 14- and 90-day studies would have been carried out at substantially higher doses. The use of higher doses would have allowed us to make a better estimate of minimal effective dosage regimens for behavioral effects.

We have also made changes in the dependent measures as a consequence of our experience with the trihalomethanes (*15,16*). A number of new measures have been added. They include photocell motor activity, body weight, food intake, a standardized behavioral observation, body temperature measurement, sensory tests (olfaction and nociception), social behavior, and taste aversion testing. In addition, unlike the case for the trihalomethanes where most testing was initiated one day after the last dose of the regimen, many of the assessments are now made throughout the exposure. We no longer use the hold-board test since we believe that photocell motor activity is assessing some of the same behavioral functions. The screen test has been modified to record latency to climb to the top of the screen rather than the nominal result of success or failure. On the other hand, few changes have been made in our approach to behavioral teratology assessment. Operant behavioral testing has been deemphasized since it lends itself best to within-subjects designs and in a screening program the large effort required may not be justified. We still find it useful to characterize maximal no effect levels by a procedure involving an ascending dosage series which continues until effects are found.

In conclusion, we have presented our evaluation of the behavioral toxicity of trihalomethane contaminants of drinking water. The purpose of these studies was twofold: to develop an approach to safety evaluation of drinking water contaminants and to provide some data on the behavioral effects of trihalomethanes. The trihalomethanes have not proven to be very active on most behavioral tests and thus regulatory decisions for these compounds are unlikely to need to focus on behavioral toxicity. Our results with the trihalomethanes, however, have been very helpful in the development of a strategy for assessment of the behavioral toxicity of drinking water contaminants.

The research reported here was supported by EPA Grant No. R804701010. Dr. Mary Jeanne Kallman joined the behavioral toxicology project after the completion of testing the trihalomethanes. She has contributed substantially to the assessment of this data and the evolution of our approach. She also critically reviewed this manuscript. The research was carried out by a group of creative and industrious laboratory technicians to whom we are indebted: J. Bernard Baird, Jack Burkhalter, Gary Crickenberger, Edward Dimen, Richard Lane, Roxanne Pross and Edwin Talley. Shirley Jessup and Bonny Hopkins assisted in the preparation of the manuscript. Lastly, the advice, encouragement and support by Drs. Robert Tardiff and Kirby Campbell of the EPA is gratefully acknowledged.

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Environmental Health Perspectives
Vol. 46, pp. 137-140, 1982

Effects of Transplacental Exposure to Chlorinated Phenols

by Jerry H. Exon* and Loren D. Koller*

Female rats were exposed to 0, 5, 50 or 500 ppm of 2-chlorophenol (2-CP) or pentachlorophenol (PCP). The study was designed to produce progeny which were exposed to the chlorophenolic compounds both prenatally and postnatally. Percent conception, litter size, birth weight, and number of stillbirths was determined at parturition. Hematologic parameters and body weights of the progeny were recorded at weaning age (3 weeks).

Effects on reproduction were observed in both the 2-CP and PCP-exposed groups, as indicated by decreased litter sizes and increased number of stillborn. The data indicate that these chlorinated phenolic compounds may be feto- or embryotoxic at high doses. Effects on hematologic parameters were not observed. Further study involving transplacental and chronic exposures to these chlorophenolic compounds appears warranted.

The chlorophenols are a group of 19 compounds which differ in degree and position of chlorination (*1*). These compounds consist of mono-, di-, tri- and tetrachloro isomers and a pentachlorophenol. The chlorinated phenols are effective disinfectants, antiseptics, fungicides, slimicides, bactericides, wood preservatives, herbicides, insecticides, and molluscicides (*2-9*). The widespread use of these chemicals in industry and agriculture has resulted in contamination of food producing animals and the environment. Inadvertent formation of 2-chlorophenol, 2,4-dichlorophenol and 2,4,6-trichlorophenol as byproducts of water chlorination represent significant sources of exposure to these compounds (*1*).

2-Chlorophenol is a commercially produced chemical used as an intermediate in the production of higher chlorinated phenols. The generation of waste sources from the commercial production of 2-chlorophenol, its chemically derived products, and the inadvertent formation of 2-chlorophenol due to chlorination of organics in drinking and waste waters are potential sources of environmental contamination (*1*).

Pentachlorophenol (PCP) and its salts have been widely used in agriculture and industry since 1936 (*10*). Approximately 200 million pounds of PCP were produced worldwide in 1977 (*11*). Principal uses of PCP are as a pesticide and wood preservative (*12*), and contamination of livestock occurs by licking treated wood or drinking from vats used to treat wood (*6*). Industrial exposure and consumption of contaminated food and water are the principal sources of exposure to humans (*13*).

This study was conducted to assess the effects of two chlorophenolic compounds, 2-chlorophenol and pentachlorophenol, on reproduction and hematology. The experimental design included prenatal and postnatal exposure to the chlorophenolic compounds.

Materials and Methods

Female Sprague-Dawley rats were weaned at 21 days of age and divided into groups of 12-20 rats each. The rats were placed on dietary regimens containing 0, 5, 50 or 500 ppm 2-CP (Aldrich Chemicals, 97% pure) in the drinking water or PCP (ICN Pharmaceuticals, K & K Laboratories No. 18497, 95% pure) in the feed. The female rats were housed four per cage in stainless hanging wire racks. Feed and water were available *ad libitum*. The rats were bred at 90 days of age (10 weeks exposure). The dams were transferred to polycarbonate cages with stainless steel lids and hardwood shavings to litter. The study was designed to produce progeny that were exposed to the chlorophenolic contaminants both prenatally and postna-

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tally. Percent conception, litter size, birth weight, and number of stillbirths was determined at parturition. Body weights and hematologic parameters (white and red cell counts, hemoglobin, packed cell volume and mean corpuscular volume) were recorded at weaning age. Blood samples were analyzed using a Coulter counter, Model ZB_i. The dams were terminated at weaning and liver and kidney tissues were collected for analysis of chlorophenolic content. Gas chromatography was used to analyze 2-CP and PCP in tissues by the methods of Erney (*14*).

Results

Parameters relating to reproductive performance that were observed include percent conception of the dams, litter size, birth and weaning weights, percent stillborn, survival to weaning and body weight gain of the dams (Tables 1–4). Percent conception was greater in all treatment groups as compared to controls (Tables 1 and 2). Litter size was significantly ($p \leq 0.05$) decreased in groups of dams treated with high levels of 2-CP (Table 1). Litter size was also decreased in groups given 500 ppm PCP ($p \leq 0.10$) (Table 2). Percent of stillborn pups born to dams receiving either 2-CP or PCP was generally greater as compared to controls. This increase was significant ($p \leq 0.05$) in the 500 ppm 2-CP group (Table 1). Body weights at weaning were generally decreased in PCP-exposed groups. No consistant effects were observed in regard to survival to weaning (Tables 3 and 4). The body weight gains of dams prior to breeding appeared to be unaffected by the treatments.

Table 1. Effect of 2-chlorophenol (CP) on rats and progeny.

Treatment[a]	Conception, %	Litter size (mean ± SD)[b]	Birth wt., g (mean ± SD)[c]	Stillborn, %	Mean weight gain of dams, g
Control	67 (8/12)	11.4 ± 1.2	2.2 ± 0.4	0 (0/91)	168
CP					
5 ppm	75 (9/12)	11.7 ± 3.5	2.3 ± 0.4	0 (2/105)	170
50 ppm	75 (9/12)	10.1 ± 2.3	2.5 ± 0.4	0 (0/91)	158
500 ppm	86 (12/14)	9.2 ± 4.3	2.4 ± 0.4	0 (6/110)	159

[a] CP was given in the drinking water of the dams from weaning through parturition.

[b] Live and stillborn.

[c] Live pups only.

[d] Significant at $p < 0.05$ to control.

Table 2. Effect of pentachlorophenol (PCP) on rats and progeny.

Treatment[a]	Conception, %	Litter size (mean ± SD)[b]	Birth wt., g (mean ± SD)[c]	Stillborn, %	Mean weight gain of dams, g
Control	58 (7/12)	10.6 ± 2.4	2.6 ± 0.7	1 (1/74)	146
PCP					
5 ppm	77 (10/13)	10.3 ± 3.2	2.5 ± 0.3	7 (7/103)	145
50 ppm	85 (11/13)	10.7 ± 3.5	2.5 ± 0.3	2 (2/110)	130
500 ppm	86 (12/14)	9.2 ± 3.0	2.4 ± 0.5	3 (3/110)	11

[a] PCP was given in the feed of the dams from weaning through parturition.

[b] Live and stillborn.

[c] Live pups only.

[d] Significant at $p < 0.10$ to control.

Table 3. Effect of 2-chlorophenol (CP) on progeny of rats.

Treatment[a]	Weaning wt. (mean ± SD), g	Survival to weaning, %[b]
Control	51.3 (4.4)	100 (91/91)
CP		
5 ppm	54.2 (8.4)	100 (103/103)
50 ppm	54.1 (8.4)	100 (91/91)
500 ppm	53.9 (9.6)	99 (103/103)

[a] 2-Chlorophenol was given in the drinking water either prenatally by exposing the dams from weaning through parturition (90 days) and postnatally from parturition.

[b] Exclusive of stillborn pups.

Table 4. Effect of pentachlorophenol (PCP) on progeny of rats.

Treatment[a]	Weaning wt. (mean ± SD), g	Survival to weaning, %[b]
Control	60.0 (9.8)	97 (71 73)
PCP		
5 ppm	56.7 (6.8)	89 (9 96)
50 ppm	55.7 (7.6)	98 (1 /108)
500 ppm	54.9 (12.2)	99 (1 /107)

[a] Pentachlorophenol was given in the feed prenatally by exposing the dams from weaning through parturition (90 days) and postnatally from parturition.

[b] Exclusive of stillborn pups.

Table 5. Analysis of feed and tissue samples of dams fed pentachlorophenol or 2-chlorophenol for 10 weeks.

	Chlorophenol content, ppm		
	Feed[a]	Liver[b]	Kidney[b]
Control (PCP)	0.21	0.048	0.066
PCP			
5 ppm	4.80	0.102	0.034
50 ppm	71.40	0.081	0.046
500 ppm	411.50	0.099	0.056
Control (2CP)	—	0.16	0.26
2CP			
5 ppm	—	2.20	2.60
50 ppm	—	3.20	2.40
500 ppm	—	0.08	2.00

[a] A pool of three separate bags.
[b] A pool of tissues from five rats/group.

Table 6. Chemical content of pentachlorophenol mixture used to prepare PCP diets.[a]

	Content
Composition by gas chromatography	
2,3,4,6-Tetrachlorophenol	6.8%
2,3,4,5-Tetrachlorophenol	0.6%
Pentachlorophenol	85.5%
Dioxins by liquid chromatography	
Hexachlorodibenzo-*p*-dioxins	8 ppm
Oxtochlorodibenzo-*p*-dioxins	400 ppm

[a] ICN Pharmaceutical, K&K Laboratories, catalog no. 18497; 97% pure.

The accumulation of the chlorophenol compounds in tissues of dams was comparatively minimal (Table 5). Pentachlorophenol levels in liver and kidney tissues were considerably lower than 2-CP residues in similar tissues. Residues in kidney versus liver tissue were approximately equal. Feed samples were also analyzed for PCF content (Table 5). The analysis correlated reasonably well with the target concentrations of 5, 50 and 500 ppm. Purity of the PCP mixture used in preparation of the PCP diet was analyzed by the Dow Chemical Company (Table 6). This analysis incidated that the PCP formulation was only 85.5% pure as compared to the 95% purity advertised by the manufacturer. The dioxin content was also determined (Table 6).

Hematologic parameters examined for all treatment groups included red and white blood cell counts, packed cell volume (hematocrit), hemoglobin and mean corpuscular volume (Tables 7 and 8). No significant effects of chlorinated phenols on hematologic parameters were observed.

Discussion

The chlorophenols tested, 2-CP and PCP, appeared to alter certain reproduction parameters in rats. Stimulated conception rates, reduced litter size at the highest doses (500 ppm), and increased number of stillbirths were observed in chlorophenol-treated groups. These results indicate that high levels of these compounds tend to be feto- or embryotoxic.

Table 7. Hematologic parameters of rats exposed to 2-chlorophenol.[a,b]

Treatment	RBC $10^4/mm^3$	Hct, % PCV	MCV, μm^3	WBC, $10^2/mm^3$	Hgb, g/dl
Control	747 (46)	37 (3)	49 (2)	163 (46)	16.1 (0.9)
CP					
5 ppm	728 (56)	36 (3)	47 (2)	164 (27)	16.2 (0.9)
50 ppm	778 (38)	40 (2)	48 (2)	166 (34)	16.5 (0.9)
500 ppm	828 (57)	42 (4)	48 (2)	178 (46)	17.5 (1.6)

[a] 2-Chlorophenol was given in the drinking water either prenatally by exposing the dams from weaning through parturition (90 days) and postnatally from parturition.
[b] All hematologic parameters are reported as mean (standard deviation).

Table 8. Hematologic parameters of rats exposed to pentachlorophenol.[a,b]

Treatment	RBC $10^4/mm^3$	Hct, % PCV	MCV, μm^3	WBC, $10^2/mm^3$	Hgb, g/dl
Control	767 (42)	39 (2)	50 (1)	162 (34)	16.2 (0.8)
PCP					
5 ppm	781 (51)	38 (2)	49 (1)	161 (32)	16.2 (0.8)
50 ppm	786 (23)	38 (2)	49 (2)	154 (28)	16.1 (0.5)
500 ppm	789 (66)	37 (2)	47 (2)	192 (57)	15.8 (1.2)

[a] Pentachlorophenol was given in the drinking water either prenatally by exposing the dams from weaning through parturition (90 days) and postnatally from parturition.
[b] All hematologic parameters are reported as mean (standard deviation).

The results are consistent with those published by others (*15*).

The majority of commercially prepared technical-grade PCP is a mixture of about 85–90% PCP and the remainder primarily consists of tetrachlorophenol and chlorinated phenoxyphenols (*16*). Small amounts of other impurities include chlorinated dibenzo-*p*-dioxins, chlorinated dibenzofurans and chlorinated diphenyl ethers. The mixture purchased for this study was listed by the manufacturer to be 95% pure PCP. However, analysis indicated that the mixture contained actually 85.5% PCP and a variety of other phenolics and impurities. Nevertheless, exposure to this substance in the environment would be as prepared containing both the PCP and associated impurities. Once adverse effects are identified from exposure to the manufactured product, then analytical grade PCP must be evaluated to determine if the effects are produced by PCP, its impurities or from a combination of these products.

This work was supported by U.S. Environmental Protection Agency grant R8072670110.

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Environmental Health Perspectives
Vol. 46, pp. 141-149, 1982

Dose-Response Study of Chloroform Carcinogenesis in the Mouse and Rat: Status Report

by Theodore A. Jorgenson,* Carol J. Rushbrook* and David C. L. Jones*

Chloroform is being administered to male Osborne-Mendel rats and to female B6C3F1 mice at concentrations of 0 (negative control), 200, 400, 900 or 1800 ppm in the drinking water. Matched control groups of both species receive a volume of water identical to that consumed by the corresponding 1800 ppm groups. At this writing, the animals have completed 23 months on test. Negative control and $CHCl_3$-treated rat groups have shown typical growth curves, with dose-related relative decrements in body weight evident throughout the study. Following decreases in $CHCl_3$ groups during the first 8 weeks, rat water consumption values have continued to increase slowly, but persistent relative dose-related decrements are evident. No initial treatment-related decrements are evident. No initial treatment-related mortality was seen in the rats. Survival is 21, 41, 45, 76, 70 and 64% for the negative control, 200, 400, 900, 1800 ppm and matched control groups, respectively. Survival values for mice at three weeks were 99, 94, 74 and 76% for the 200, 400, 900 and 1800 ppm groups, respectively. Mortality was apparently related to markedly decreased fluid consumption among some of the treated mice. Subsequent mortality has been less than 15% for all mouse groups. Except for acclimation effects during the first 2-3 weeks, body weights for the treated mouse groups have been generally within 10% of negative control values. Tissue changes in decedents have been similar in treated and control groups, both in rats and mice. Terminal sacrifice and histologic evaluations will be initiated after completion of 24 months on test.

Introduction

There have been several studies of tumorigenesis induced in animals by chloroform. Eschenbrenner (*1*) reported hepatomas induced in female Strain A mice receiving 0.3 or 0.6 mg/kg of chloroform by gavage every four days for three months. In a study conducted by Hazleton Laboratories in 1976 for the National Cancer Institute (*2*), chloroform was administered by gavage five times per week for 78 weeks to Osborne-Mendel rats at 90–250 mg/kg/day and to B6C3F1 mice at 100–500 mg/kg/day. Kidney epithelial tumors were induced in male rats and hepatocellular carcinomas in mice of both sexes. Decreased survival rates and body weights were reported in treated rats but were not observed in the mice. Theiss et al. (*3*) reported no increase in lung adenomas in Strain A mice receiving intraperitoneal injections of chloroform at 80 or 200 mg/kg three times per week for 8 weeks, or two injections at 400 mg/kg.

The present study was designed to evaluate the chronic toxicity and tumorigenicity of chloroform administered in the drinking water to male Osborne-Mendel rats and to female B6C3F1 mice over a wide range of dose levels.

Methods

Animals

The species and strains of rodents used were those in the earlier study conducted by Hazleton. Rats were received from CAMM Research, and mice from Charles River. Mice were housed five

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per cage in 48.3 × 26.7 × 15.2 cm (19 × 10.5 × 6 in.) polycarbonate cages containing hardwood chip bedding (AbSorbDri). Cages were changed once a week at the same time as the body weights and water consumption were recorded.

Rats were housed two per cage in 43.2 × 21.6 × 20.3 cm (17 × 8.5 × 8 in.) polycarbonate cages containing the same hardwood chip bedding. Cages were changed twice a week: once when body weights and water consumption were recorded and again when water consumption was recorded for the second time in the week and the cages and racks were rotated.

Animals were assigned to experimental groups and cages using a table of random numbers.

For both species cages were rotated on the racks once a week. The racks were rotated on the same schedule within the room to ensure similar conditions for all animals throughout the study.

Control animals were housed in rooms as similar as possible but separate from the chloroform-treated groups.

Chemical Supply

The chloroform used in this program was pesticide quality purchased from Matheson Coleman Bell. The chloroform was analyzed for purity, concentration and stability in the drinking water. Before use, all chloroform was distilled to remove contaminants (diethyl carbonate, ethanol, and phosgene). Distilled water was used throughout the study for control drinking water as well as for the chloroform solutions.

Analytical Chemistry

Quantitative analyses for chloroform in air, water, feed and blood; distillation of chloroform and determination of impurity levels; determination of liver fat/organ weight ratios; and routine monitoring of feed lots for PCBs and chlorinated pesticides were performed. Ambient air in each experimental room was analyzed once every 6 months for chloroform concentrations. The Purina Laboratory Chow was analyzed annually for chloroform concentration with each shipment being analyzed for PCBs and chlorinated pesticides. Chloroform in blood from additional rats placed on test for this purpose was analyzed at 3, 6, 12, and 18 months and will be evaluated at 24 months on study. Once each month the stock solutions prepared on Tuesday were analyzed for chloroform. Additionally, samples from water bottles were analyzed once each month on the Friday following the stock solution analysis. Liver fat/liver weight ratios were examined at 3 and 6 months in both rats and mice.

Dose Levels and Number of Animals

The negative control (0 ppm) and 200 ppm rat groups were each composed of 330 animals. There were 150 rats in the 400 ppm group and 50 in the 900 ppm, 1800 ppm and matched control (50) groups. The negative control and 200 ppm mouse groups each had 430 animals, while the numbers of mice in the other groups were identical to the corresponding rat groups.

In addition to the above numbers of animals, there were 80 additional male rats per group for blood work and 10 additional male rats in all but the matched control group for liver fat analyses at 90 days on test. With the exception of the matched control group, each of the mouse groups had 20 additional mice. Ten mice per group were removed at 3 months and at 6 months for liver fat analyses.

Observations

Food intake and animal health and activity were noted daily, 7 days a week. Body weights were recorded weekly until stable (18 weeks for the rats and 14 weeks for the mice). Thereafter, body weight measurements were recorded monthly. Water consumption was measured twice weekly by weighing the water bottles during the two changes in water each week.

Hematology and Blood Chemistry

Blood parameters listed in Table 1 were evaluated on 20 male rats from each experimental group at 6, 12, and 18 months, and will be evaluated at 24 months on test.

A Coulter Counter Model S was used to determine red and white cell counts, hemoglobin, hematocrit, MCV, MCH, and MCHC. Differential counts were conducted manually. The chemical analyses were run on a Sequential Multiple Analysis Plus Computer (SMAC) system.

These additional animals were handled in the same manner as those in the lifetime study. At the time of sacrifice for blood analyses, we performed a gross necropsy and collected and saved the appropriate tissues in 10% neutral buffered formalin.

Gross and Microscopic Pathology

When any animal in these lifetime studies appeared unlikely to survive until the next scheduled observation, it was sacrificed. This aggressive sacrifice was intended to prevent the loss of information that could occur through autolysis or cannibalization.

All decedents were given a complete gross nec-

Table 1. Blood parameters evaluated.

Hematology	Clinical chemistry	
Hematocrit	Triglycerides	Glucose
Hemoglobin	Total bilirubin	BUN
Red cell count	SGOT	Creatinine
White cell count	SGPT	Uric acid
Differential count	LDH	Na^+
MCV	Alkaline phosphatase	K^+
MCH	Total iron	CO_2
	Total protein	Cl^-
MCHC	Albumin	Calcium
	Globulin	Phosphorus
	A/G ratio	Balance ($Na-(Cl + CO_2)$)
		Cholesterol

Table 2. Measured chloroform concentrations in water bottles in percent of nominal values.[a]

Month	Percentage of nominal values at various chloroform levels			
	200 ppm	400 ppm	900 ppm	1800 ppm
1	93	94	88	76
2	84	93	95	94
3	104	108	80	94
4	92	84	89	89
5	99	102	100	99
6	106	94	90	73
7	106	100	101	98
8	107	97	96	98
9	103	104	105	103
10	104	104	100	94
11	95	96	91	88
12	89	88	88	83
13	96	98	94	97
14	90	95	92	89
15	100	104	100	97
16	108	100	100	105
17	100	103	121	96
18	91	87	95	94
19	104	102	101	98
20	104	104	101	105
21	104	100	107	96
22	92	93	90	79
23	105	100	110	109
24	117	111	109	99

[a] Mean of [illegible] samples per level per month.

ropsy as defined in the Guidelines for Carcinogen Bioassay in Small Rodents, DHEW Publication No. (NIH) 78 [illegible]3, Revised 1978, including examination of external surfaces and body orifices, and examination and fixation of all of the following: gross lesions, tissue masses or suspect tumors and regional lymph nodes, skin, mandibular lymph node, mammary gland, salivary gland, larynx, trachea, cecum, colon, rectum, mesenteric lymph node, liver, thigh muscle, lungs and bronchi, heart, thyroids, parathyroids, esophagus, stomach, duodenum, jejunum, ileum, spleen, kidneys, adrenals, bladder, seminal vesicles, prostate, sciatic nerve, sternebrae or vertebrae, or femur (plus marrow), costochondral junction, ribs, thymus, gall bladder, pancreas, testes, ovaries, uterus, nasal cavity, brain, pituitary, eyes and spinal cord. All tissues and organs were fixed in 10% neutral buffered formalin.

The following tissues were processed and read histologically, with all others being stored in fixative: suspect tumors and gross lesions, liver, kidney, regional lymph nodes, urinary bladder, esophagus, adrenals, spleen, stomach, small intestine, colon and lung. All rat slides were read by one pathologist, and all mouse slides were read by another pathologist.

Results

Since this study is still in progress, the results presented here are necessarily incomplete. Nonetheless, they provide a basis for evaluating the progress of the study and some information about the chronic effects of chloroform.

Table 2 shows the results of the analyses of the chloroform solutions in the drinking bottles. Chloroform concentrations varied somewhat over the course of the study, but were generally within ten percent of the nominal values. Clearly, there were no overlapping values between adjacent dose groups.

Survival, water consumption, and body weight patterns for the rats are shown in Figure 1. Survival has been essentially directly proportional to the chloroform dose level; lowest for the negative controls, next lowest for the 200 and 400 ppm groups, and highest for the matched controls and the 900 and 1800 ppm groups. Water consumption has increased slowly over the course of the study in all groups, with the amount consumed inversely proportional to dose level during the first 18 months, then a clustering of the negative control, 200 and 400 ppm groups at a relatively higher level, the 900 ppm group in the middle, and the 1800 ppm group at a lower level. Body weight patterns have shown typical growth curves, the values at any time point being inversely proportional to dose level. Values for the matched control group were above those for the 1800 ppm group during the first year, but the latter group has now caught up with its matched controls.

Survival, water consumption and body weight patterns for the mice are illustrated in Figure 2. Unlike the rats, there was significant early mortality in the treated mice, particularly at 900 and 1800 ppm. From the second through the eighteenth month very few animals died, but by the twenty-third month some mortality, proportional to dose level,

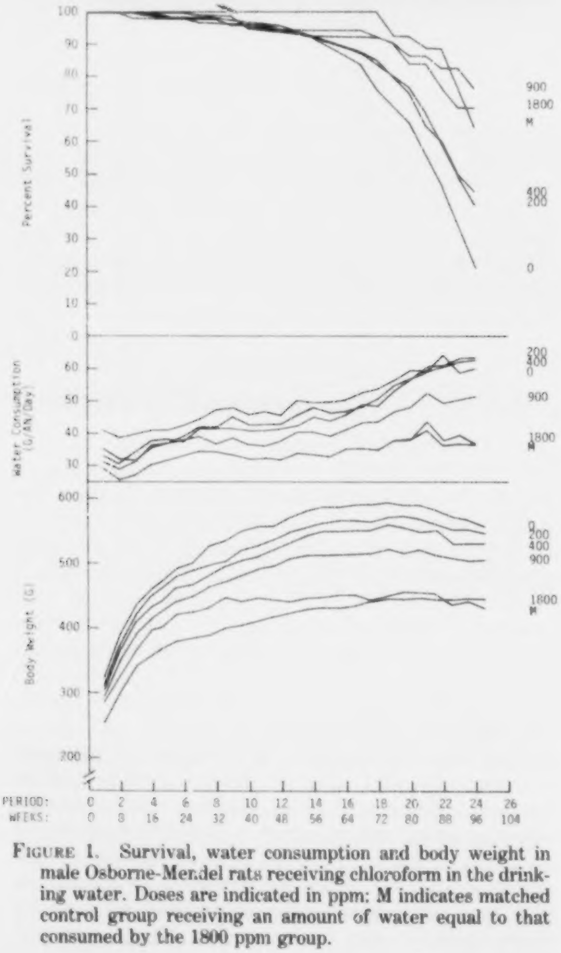

FIGURE 1. Survival, water consumption and body weight in male Osborne-Mendel rats receiving chloroform in the drinking water. Doses are indicated in ppm; M indicates matched control group receiving an amount of water equal to that consumed by the 1800 ppm group.

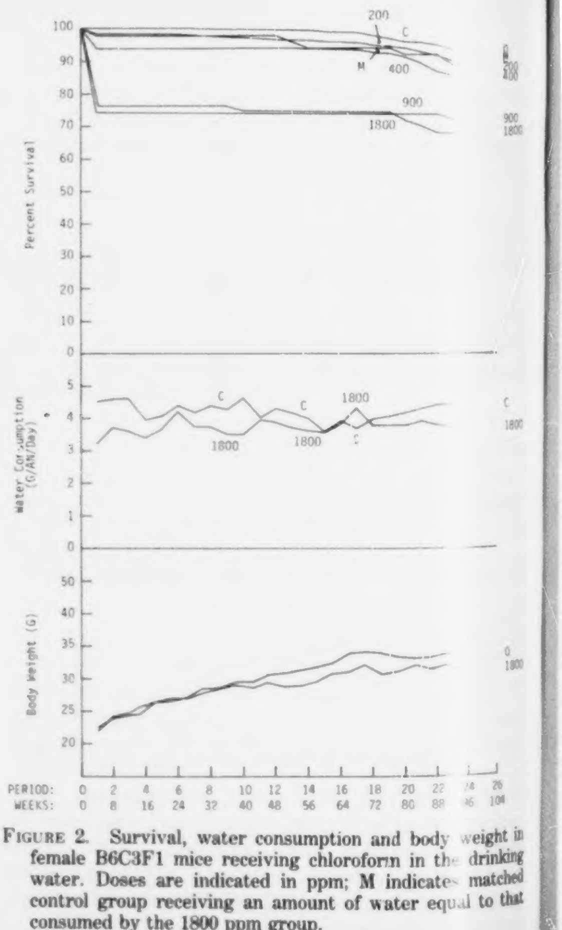

FIGURE 2. Survival, water consumption and body weight in female B6C3F1 mice receiving chloroform in the drinking water. Doses are indicated in ppm; M indicates matched control group receiving an amount of water equal to that consumed by the 1800 ppm group.

Table 3. Mean percent liver fat in animals receiving chloroform in the drinking water.

	Dose group	3 Months		6 Months	
		N	%	*N*	%
Rats	0 ppm	10	3.3	20	4.5
	M	—	—	18	4.6
	200 ppm	10	3.3	20	4.5
	400 ppm	10	3.2	20	4.6
	900 ppm	10	3.6	19	4.8
	1800 ppm	10	3.5	19	5.1[a]
Mice	0 ppm	10	3.3	7	5.8
	200 ppm	10	3.5	10	7.9[a]
	400 ppm	10	3.9[b]	10	6.8
	900 ppm	10	4.5[b]	6	7.1[a]
	1800 ppm	8	6.4[b]	8	10.4[b]

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

was apparent. Both water consumption and body weights were narrowly distributed among the various groups. The values for the two extreme groups, the negative controls and the 1800 ppm group, are shown for illustration. Water consumption for the mice was essentially stable throughout the study. Body weight increased more or less linearly, then reached a plateau beginning at about 18 months.

Although mean blood levels of chloroform were assessed in the rats at 3, 6, 12 and 18 months, there was difficulty in obtaining reliable data during the first year. For the 18-month assay, whole blood was used instead of serum, and reliable results were obtained. Values were 0.7, 0.8, 7.5, 22.1, 75.4 and 124 ppb for the negative controls, matched controls, and the 200, 400, 900 and 1800 ppm groups, respectively.

The percent fat in the liver of the rats and the mice at 3 and at 6 months is shown in Table 3. For the rats, there was no apparent increase in liver fat content in the treated groups at 3 months, but at 6 months there was a significant increase in the 1800 ppm group. In the mice, significant increases in liver fat were apparent at 400-1800 ppm at 3 months and at 900 and 1800 ppm at 6 months.

The hematologic findings in the rats at 6, 12 and 18 months are shown in Tables 4–6. WBC values were lower in the 1800 ppm group and in the matched controls at 6 and at 12 months. The differences in erythrocyte and hemoglobin parameters at 12 months are consistent with hemoconcentration in the treated groups, but no significant differences were apparent at 18 months.

The blood chemistry data for male rats sacrificed at 6, 12 and 18 months are shown in Tables 7–9. Although various parameters differed significantly from the negative control values at some time points, there were a number of apparent trends. Chloride,

Table 4. Summary of hematology data for male Osborne-Mendel rats receiving chloroform in their drinking water: 6-month sacrifice.

Parameter	Control	Matched control	Chloroform 200 ppm	400 ppm	900 ppm	1800 ppm
WBC × 10^3/mm^3	6.8	4.8[a]	6.6	6.5	6.8	5.5[a]
RBC × 10^6/mm^3	8.06	8.14	8.19	8.07	8.04	7.98
Hgb, g-%	15.3	15.4	15.5	15.3	15.2	15.3
Hct, %	44.2	44.3	44.4	43.6	43.7	44.0
MCV, μ^3	55	55	54	54	55	55.
MCH, $\mu\mu$g	18.9	18.7	18.8	18.7	18.8	19.0
MCHC, %	34.7	34.7	35.0	35.0	34.9	34.8
Differential						
PMN,%	10.7	14	13	12	11	12
Bands, %	0	0.1	0.1	0	0.1	0
Lymphocytes, %	85	82	84	85	86	84
Monocytes, %	2.5	2	2	2	2	2
Eosinophils, %	2	2	1.6	1	1	2
Basophils, %	0	0	0	0	0	0

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

Table 5. Summary of hematology data for male Osborne-Mendel rats receiving chloroform in their drinking water: one-year sacrifice.

Parameter	Control	Matched control[a]	Chloroform			
			200 ppm	400 ppm	900 ppm	0 ppm
WBC × 10^3/mm^3	6.7	5.9^b	6.6	6.4	5.0^b	4.4^b
RBC × 10^6/mm^3	7.84	7.98	8.17^b	7.97	8.31^b	7.97
Hgb, g-%	14.9	15.2	15.3^c	15.2	15.6^b	15.4^c
Hct, %	43.1	44.6^c	43.4	44.4	45.1^b	44.4^c
MCV, μ^3	55	56	53^b	56	55	56
MCH, $\mu\mu$g	18.9	18.9	18.7	19.0	18.7	19.2
MCHC, %	34.4	33.9	35.3^c	34.0	34.5	34.5
Differential						
PMN,%	20	19	16	14	17	16
Bands, %	0	0	0	0	0	0
Lymphocytes, %	78	79	81	84	80	81
Monocytes, %	2	1	1	1	2	1
Eosinophils, %	1	1	1	1	2	2
Basophils, %	0	0	0	0	0	0

[a] One sample clotted, therefore, only 19 samples were analyzed for this level. All other levels contained 20 samples. The differential count for this level contained 20 samples.

[b] $p \leq 0.01$.

[c] $p \leq 0.05$.

Table 6. Summary of hematology data for male Osborne-Mendel rats receiving chloroform in their drinking water: 18-month sacrifice.

Parameter	Control	Matched control[a]	Chloroform			
			200 ppm	400 ppm	900 ppm	1800 ppm
WBC × 10^3/mm^3	6.6	6.2	6.0	6.7	5.8	6.4
RBC × 10^6/mm^3	7.43	7.57	7.38	7.66	7.76	7.49
Hgb, g-%	14.4	14.7	14.4	15.0	14.9	14.4
Hct, %	41.6	42.8	41.4	42.8	42.5	41.5
MCV, μ^3	56	57	57	56	55	56
MCH, $\mu\mu$g	19.4	19.4	19.5	19.6	19.2	18.7
MCHC, %	34.8	34.3	34.7	35.2	35.0	34.7
Differential						
PMN,%	21	21	19	21	20	27
Bands, %	0	0	0	0	0	0
Lymphocytes, %	74	76	77	75	77	69
Monocytes, %	3	2	2	3	2	2
Eosinophils, %	2	2	2	1	1	2
Basophils, %	0	0	0	0	0	0

[a] One sample clotted; therefore, only 19 samples were analyzed for this level. All other levels contained 20 samples. The differential count for this level contained 20 samples.

potassium, phosphorus, bilirubin, alkaline phosphatase, total iron, albumin and the albumin/globulin ratio tended to be higher in treated groups than in the negative controls. Cholesterol, triglycerides, LDH and globulin tended to be lower in treated groups than in the negative controls. In the matched controls, glucose and total iron tended to be higher and cholesterol and triglycerides tended to be lower than in the negative controls. In summary, most of the changes in hematologic and blood chemistry parameters observed in the treated groups were also evident in the matched control group. Thus, these effects of chloroform appear to be secondary to reduced water and food consumption.

Histopathologic evaluations have been completed for 200 rats and 62 mice that were sacrificed or found dead during the first 20 months of the study. From the mortality data, it is apparent that these samples are highly biased both in time and toward negative control animals. The percentages of rats examined to date are 45, 12, 10, 5, 6 and 4% for the negative control, matched control, 200, 400, 900 and 1800 ppm groups, respectively. Corresponding percentages for the mice are 3, 4, 4, 6, 20 and 18%

Table 7. Summary of clinical chemistry data for male Osborne-Mendel rats receiving chloroform in their drinking water: 6-month sacrifice.

Parameter	Control	Matched control	Chloroform			
			200 ppm	400 ppm	900 ppm	1800 ppm
Glucose, mg-%	182	215^{a}	180	187	201	193
BUN, mg-%	22	24.4^{b}	21.6	20.6	21.2	26.3^{b}
Creatinine, mg-%	0.6	0.6	0.6	0.6	0.7	0.7^{a}
Uric acid, mg-%	2.0	2.9	2.1	2.1	2.5	2.9
Na^+, meq/l	146	147	146	146	146	145
K^+, meq/l.	5.3	5.9	5.2	5.4	5.6	6.4^{b}
CO_2, meq/l	24.0	24.8	26.6	27.0	26.0	26.0
Cl^-, meq/l.	99.7	96.3	99.7	100	102^{b}	102^{b}
Calcium, mg-%	10.0	10.0	9.9	10.1	10.3^{b}	10.5^{b}
Phosphorus, mg-%	5.9	5.8	6.0	6.1	6.7^{b}	7.4^{b}
Blance, Na-(Cl + CO_2)	22	20	20	18.5^{b}	18^{b}	18^{b}
Cholesterol, mg-%	111	89^{a}	107	114	103	100
Triglycerides, mg-%	127	69^{b}	120	116	68^{b}	32^{b}
Total bilirubin, mg-%	0.13	0.13	0.13	0.13	0.17^{a}	0.19^{b}
SGOT, mU/ml	180	170	135	127	114^{a}	129
SGPT, mU/ml	114	101	81	82	47	91
LDH, mU/ml	1828	1169^{b}	1195^{b}	994^{b}	821^{b}	744^{b}
Alkaline phosphatase, mU/ml	194	221	180^{a}	173	165	197
Total iron, µg-%	182	193	170	188	209^{b}	202
Total protein, g-%	5.7	5.9	5.6	5.6	5.7	5.7
Albumin (A), g-%	2.8	3.0^{a}	2.8	2.8	3.0^{b}	3.1^{b}
Globulin (G), g-%	2.8	2.9	2.8	2.8	2.7^{a}	2.6^{b}
A/G	1.0	1.05	1.0	1.05	1.1^{b}	1.2^{b}

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

Table 8. Summary of clinical chemistry data for male Osborne-Mendel rats receiving chloroform in their drinking water: one-year sacrifice.

Parameter	Control	Matched control	Chloroform			
			200 ppm	400 ppm	900 ppm	1800 ppm
Glucose, mg-%	182	240^{a}	183	220	220	221
BUN, mg-%	22	23	20^{b}	21	21	24^{b}
Creatinine, mg-%	0.7	0.8	0.7	0.8	0.7	0.8
Uric acid, mg-%	2.3	3.1	2.0	3.5	3.5	3.1
Na^+, meq/l	146	149^{b}	146	147	146	146
K^+, meq/l	5.1	6.3^{a}	5.1	6.6^{b}	6.6	7.0^{a}
CO_2, meq/l	27	25	27	25	25	24^{a}
Cl^-, meq/l.	99	103^{b}	101^{b}	99	101^{b}	103^{b}
Calcium, mg-%	10.6	10.5	10.4	10.8	10.9	10.7
Phosphorus, mg-%	5.4	6.1^{a}	5.2	6.3^{b}	6.1^{a}	6.7^{b}
Balance, Na-(Cl + CO_2)	20	21	18^{a}	22	21	19
Cholesterol, mg-%	159	112^{b}	146	169	127^{b}	105^{b}
Triglycerides, mg-%	323	138^{b}	257	249	91^{b}	34^{b}
Total bilirubin, mg-%	0.1	0.1	0.1	0.1	0.2^{b}	0.2^{b}
SGOT, mU/ml	204	211	136^{a}	221	166	163
SGPT, mU/ml	112	117	100	156	145	105
LDH, mU/ml	1386	1380	1065^{b}	1169	1059^{a}	668^{b}
Alkaline phosphatase, mU/ml	167	148^{a}	161	140^{b}	174	218^{b}
Total iron, µg-%	164	193^{b}	181^{a}	183^{a}	213^{b}	231^{b}
Total protein, g-%	5.8	5.9	5.9	5.9	6.0	6.0
Albumin (A), g-%	2.6	2.8	2.7	2.4	2.8^{a}	3.0^{a}
Globulin (G), g-%	3.2	3.1	3.2	3.4^{b}	3.2^{b}	3.0^{b}
A/G	0.8	0.9	0.9	0.7^{a}	0.8	1.0^{b}

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

Table 9. Summary of clinical chemistry data for male Osborne-Mendel rats receiving chloroform in their drinking water: 18-month sacrifice.

Parameter	Control	Matched control	Chloroform			
			200 ppm	400 ppm	900 ppm	00 ppm
Glucose, mg-%	185	213	177	169	195	178
BUN, mg-%	32	24	36	26	22[a]	25
Creatinine, mg-%	0.8	0.7	0.9	0.7[a]	0.7[b]	0.7
Uric acid, mg-%	1.9	2.9[a]	2.1	2.0	2.2	2.2
Na^+, meq/l.	148	148	148	149	148	148
K^+, meq/l.	5.1	6.0	5.3	5.0	5.1	6.2[b]
CO_2, meq/l.	29	27	30	30	29	29
Cl^-, meq/l.	102	102	100[a]	101	102	104[a]
Calcium, mg-%	10.3	10.2	10.5	10.2	10.2	10.1
Phosphorus, mg-%	5.2	5.5	5.2	4.9	5.3	6.4[b]
Balance, Na-(Cl + CO_2)	17	19	18	18	17	15
Cholesterol, mg-%	233	157[b]	231	200	157[b]	121[b]
Triglycerides, mg-%	375	225	373	255	130[b]	41[b]
Total bilirubin, mg-%	0.1	0.1	0.1	0.1	0.1	0.1
SGOT, mU/ml	99	153[b]	106	99	122	92
SGPT, mU/ml	61	98[b]	70	68	82	82[a]
LDH, mU/ml	944	1005	1028	854	778	560
Alkaline phosphatase, mU/ml	122	170[b]	119	124	135	167[b]
Total iron, μg%	119	165[b]	147[a]	140[a]	161[b]	162[b]
Total protein, g-%	5.4	5.5	5.5	5.4	5.5	5.4
Albumin (A), g-%	2.2	2.3	2.2	2.2	2.5[b]	2.6[b]
Globulin (G), g-%	3.8	3.1	3.3	3.2	3.0[a]	2.8[b]
A/G	0.7	0.7	0.7	0.7	0.8[b]	0.9[b]

[a] $p \leq 0.05$.
[b] $p \leq 0.01$.

(including early deaths). Thus, assessment of the effect of chloroform on tumorigenesis is not possible at this time.

For the rats, the conditions listed in Table 10 have been observed in appreciable numbers in the indicated sites.

In addition, sporadic tumor diagnoses have been made in the rats in various organs, including disseminated lymphosarcoma; adenocarcinoma in the GI tract; squamous cell carcinoma, undifferentiated sarcoma, and sebaceous gland adenoma in the ear; Schwannoma in the eye; fibrosarcoma in the heart; tubular carcinoma, clear cell adenoma, adenocarcinoma, transitional cell carcinoma, and hemangiosarcoma in the kidney; bronchiolar adenoma in the lung; osteosarcoma; fibroma, sarcoma, fibrosarcoma, fibroadenoma, and hemangiosarcoma in various body regions; squamous cell carcinoma in the nostril; carcinoma in the nasal cavity; parathyroid adenoma; basophilic carcinoma in the pituitary; adenocarcinoma in the prostate; squamous cell carcinoma and papilloma in the stomach; interstitial cell tumor in the testes; and carcinoma, cystadenoma, and adenoma in the thyroid.

There have been insufficient mice examined to identify any lesions of appreciable incidence. Sporadic tumors in the mice have been diagnosed as disseminated lymphoma, both histiocytic and lymphocytic; pheochromocytoma; alveolar/bronchiolar adenoma and carcinoma; mammary adenoma and adenocarcinoma; carcinoma and teratoma in the ovary and leiomyosarcoma in the uterine cervix.

Table 10. Conditions observed.

Site	Conditions
Adrenal cortex	Adenoma, hyperplasia, hypertrophy, vacuolation
Adrenal medulla	Pheochromocytoma, hyperplasia
Kidney	Cyst, calculi, glomerulonephritis, hydronephrosis
Lung	Arterial hypertrophy, atelectasis, congestion, interstitial pneumonia
Lymph nodes	Hemorrhage
Parathyroids	Hyperplasia
Spleen	Hematopoiesis, hemosiderosis
Stomach	Mineralization

Discussion

With administration of chloroform at constant levels in the drinking water, the daily doses received by the animals are a function of fluid consumption and body weight. Using the water consumption and body weights for the first and twenty-third months for illustration, the calculated mean daily doses for the rats receiving 200, 400, 900 or 1800 ppm of chloroform were 34, 66, 143 and 305 mg/kg and 34, 69, 132 and 238 mg/kg, during months 1 and 23, respectively. For the mice, the corresponding values were 34, 95, 207 and 382 mg/kg and 31, 63, 150 and 309 mg/kg. Thus, the daily doses in the present study were comparable to those used in two previous studies (*2,3*) at the higher levels and extended below them at the lower levels.

If it is assumed that the water consumption and body weight data provide indices of food consumption, the increased survival and decreased body weight in rats receiving chloroform are consistent with the well-known increase in longevity associated with caloric restriction (*4*). Since caloric restriction also results in reduced age-specific incidences of most pathologic conditions associated with aging (*4*), it will be intresting to consider the pathologic findings from the present study when they become available. It is also evident that inclusion of matched controls in long-term studies is extremely important.

In the mice the effect of chloroform on survival was markedly different than that in the rats. It appears that some of the mice rejected the chloroform solutions during the first weeks to such a degree that they were unable to survive. In fact, the only behavioral effect of the chloroform that was discernible during this study was the unthrifty appearance (e.g., lack of vigor, lassitude) of the mice during this time. From the water consumption and body weight data, it is clear that caloric restriction in the mice surviving the first weeks occurred to a much lesser extent than in the rats, if at all.

The increase in liver fat content for the mice was clearly greater than for the rats. It is probable that, in addition to differences between the mouse and the rat in the metabolism of chloroform (*5, 6*), the greater caloric restriction in the rats may have been a factor in reducing the relative accumulation of fat in the liver.

While the trends observed in some of the blood chemistry parameters associated with chloroform administration in the rat may be related to a nephrotoxic effect of the chloroform, nephropathy is one of the findings in the negative controls, and no definitive statements about the nephrotoxic effects of chloroform in the present study can be made at this time.

Discussion of the gross necropsy and histologic information will have be delayed until the terminal sacrifices and the histopathologic evaluations have been completed.

This study has been supported by EPA Contract 68-03-2616.

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Environmental Health Perspectives
Vol. 46, pp. 151-156, 1982

Trihalomethanes as Initiators and Promoters of Carcinogenesis

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Chloroform and other trihalomethanes are contaminants of drinking water that have been demonstrated to be carcinogenic in laboratory animals. Determination of the mechanism of carcinogenicity of chloroform is required so that the animal data can be extrapolated to estimate the human health hazard. The extent of the binding of chloroform to rat liver and kidney DNA was approximately 0.1% the level of binding found for dimethylnitrosamine. Neither chloroform nor bromoform, in contrast to diethylnitrosamine-initiated GGTase-positive foci in either intact or partial hepatectomized rats, promoted with phenobarbital. Tumor-promoting activity of chloroform was indicated by the slight significant increase, compared to untreated controls, in the incidence of GGTase-positive foci in rats initiated with diethylnitrosamine (DENA) followed by the administration of chloroform twice weekly for a total of 15 doses. In this study, rats administered only the DENA or the chloroform did not contain an increased incidence of GGTase-positive foci compared to untreated controls. However, the incidence of foci in the group that received DENA followed by chloroform was not statistically different from that in either the group that received only the DENA or only the chloroform. In conclusion, we were unable to demonstrate tumor-initiating activity for chloroform, and the tumor-promoting activity of chloroform indicated by our results requires further confirmation.

Introduction

The chlorination of drinking water can represent a major exposure to humans of trihalomethanes including bromoform and chloroform (*1-3*). Chloroform induced hepatocellular carcinomas and kidney epithelial tumors in mice and rats, respectively (*4*). The extrapolation of these results from animals to the estimation of the carcinogenic risk in humans of chloroform in drinking water requires the determination of the mechanism of action for chloroform carcinogenicity.

Chemicals can increase the incidence of cancer by two distinct mechanisms: genetic and epigenetic (*5,6*). The proposed genetic mechanism of chemical carcinogenesis results from the covalent reaction of the carcinogen with DNA. This alteration could produce a somatic mutation resulting in the formation of a clone (focus) of transformed cells. Thus genetic carcinogens initiate the neoplastic progression. The other mechanism of action for carcinogens is epigenetic and involves an alteration in the control of cellular differentiation and replication. Tumor promoters are epigenetic carcinogens that decrease the time required for the appearance and increase the incidence of tumors that were either spontaneously or chemically initiated. Some carcinogens act by both genetic and epigenetic mechanisms. In this paper, we have attempted to determine whether chloroform increased the incidence of cancer in the NCI bioassay by genetic, epigenetic or both mechanisms.

Materials and Methods

Animals and Chemicals

Male (225 to 275 g) Sprague-Dawley rats and female B6C3/F1 mice (8 to 9 weeks old) were purchased from Charles River (Portage, Mich.) and used throughout these studies. The animals were maintained in accordance with the standards set

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forth by the National Research Council (*7*). Unless otherwise noted, Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and distilled water were provided *ad libitum*.

Chloroform (glass-distilled, non preservative) was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.), bromoform from Tridom Chemical Co. (Hauppauge, N.Y.), diethylnitrosamine (DENA) from Eastman Kodak Co., (Rochester, N.Y.), sodium barbital from Fisher Scientific Co. (Pittsburgh, Pa.), sodium phenobarbital from J.T. Baker Chemical Co. (Phillipsburg, N. J.) and Tricaprylin from ICN Nutritional Biochemicals (Cleveland, Oh.).

DNA Binding

Groups of mice and rats were administered intragastrically bromoform-^{14}C (2.7 mCi/mmole, ICN Pharmaceutical, Inc., Irvine, Calif.), or chloroform-^{14}C (15 mCi/mmole, ICN Pharmaceutical, Inc., Irvine, Calif.) dissolved in corn oil or dimethylnitrosamine-^{14}C (50.9 mCi/mmole, New England Nuclear, Boston, Mass.) in saline. The animals were sacrificed by cervical dislocation 16-18 hr later for the trihalomethanes and after 2 hr for dimethylnitrosamine (DMN). The liver and kidney were excised and stored at -80°C until the DNA was isolated.

DNA was isolated from the liver by a modification of the procedure described by Kirby (*8*). The liver was homogenized in a lysing medium containing 1% sodium triisopropylnaphthalenesulfonate, 6% isobutanol, 6% sodium *p*-aminosalicylate, 1% sodium chloride and 0.01% sodium deoxycholate and incubated with proteinase K for 1 hr. After extraction with Kirby's phenol solution, the DNA was precipitated with 2-ethoxyethanol. The DNA was then sequentially treated with RNase, α-amylase and pronase followed by a second phenol extraction. After precipitation with 2-ethoxyethanol, the DNA was dissolved in water. For determination of radioactivity, an aliquot of the DNA was hydrolyzed in 0.1*N* HCl at 70°C for 30 min, dissolved in ACS (American Corp., Arlington Heights, Ill.) and counted in a Beckman 9000 liquid scintillation counter (Beckman Instrument Co., Palo Alto, Calif.). DNA concentration was determined by the procedure of Kissane and Robins (*9*).

The DNA was further purified by CsCl isopyknic centrifugation. The DNA was dissolved in 1.3 ml of a 0.25% SDS solution and layered onto a 3.5 ml CsCl solution of an average density of 1.80 g/cm^3 so that the final average density was 1.59 g/cm^3. Centrifugation was performed in a Beckman SW 50.1 rotor (Beckman Instrument Co., Palo Alto) at 35,000 rpm and for 65 hr. Fractions were collected by puncturing the bottom of the centrifuge tube and were monitored for radioactivity, absorbance at 260 nm and density.

In attempts to isolate the adducts of chloroform covalently bound to DNA, the DNA was hydrolyzed in 0.1*N* HCl at 70°C for 30 min. The hydrolyzate was chromatographed on a high performance liquid chromatograph equipped with a semipreparative Partisil 10 SCX ion-exchange column (Whatman Inc., Clifton, N.J.). Elution was accomplished at a flow rate of 4 ml/min with 0.025*M* ammonium phosphate (pH 4.0) for 20 min, followed by a linear increase over 20 min in the concentration so that the final concentration was 0.25*M* ammonium phosphate (pH 4.0).

Initiation Assay

The rat liver foci bioassay (*10-12*) was used to distinguish the initiation and promotion activity of trihalomethanes. The protocols of the initiation and promotion assays are depicted in Figure 1. For the initiation assay, male rats received a 2/3 partial hepatectomy while under ether anesthesia. At 20-22 hr later, the rats were administered by gavage bromoform (0.8 mmole/kg body weight) or chloroform (1.5 mmole/kg body weight) in tricaprylin or diethylnitrosamine (0.5 mmole/kg body weight) in distilled water. The chemicals administered by gavage were given as 2 ml/kg body weight. Three days later, the rats started to receive 500 ppm sodium phenobarbital in their drinking water for a total of 47 days. Six days after the termination of the phenobarbital treatment the animals were sacrificed by decapitation and the right lateral liver lobe quickly excised. Four tissue blocks, each approximately 10 × 10 × 2 mm, were taken and frozen in O.C.T. compound (Fisher Scientific Co., Pittsburgh, Pa.) on dry ice. The slices were arranged so that the leading face of each block was not serially related to the leading face of another block. The tissue blocks were stored at -80°C until being sliced at -25°C into 6 μm sections. The sections were stained by the procedure of Rutenburg et al. (*13*) for γ-glutamyl transpeptidase (GGTase) activity and counterstained with hematoxylin. GGTase-positive foci that contained nine or more nuclei were counted. From each animal at least 2 cm^2 of tissue were scanned for foci. An increase incidence of GGTase-positive foci was indicative of carcinogenic activity.

Promotion Assay

Male rats were administered by gavage an initiating dose of diethylnitrosamine (0.5 mmole/kg body weight) in distilled water (2 ml/kg body weight). Three days later, some of the rats received intragastrically chloroform (1.5 mmole/kg body weight)

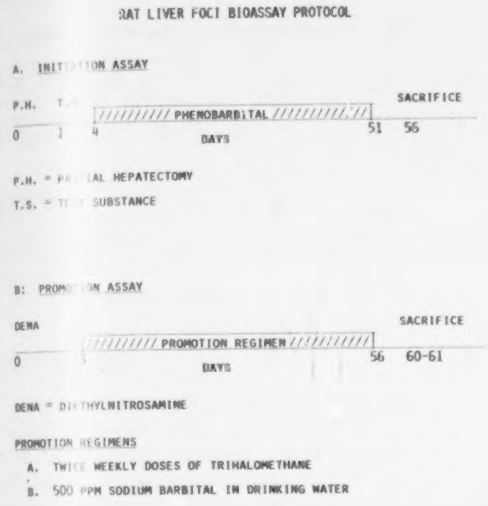
RAT LIVER FOCI BIOASSAY PROTOCOL
A. INITIATION ASSAY
P.H.
T.S.
SACRIFICE
PHENOBARBITAL
0
1
4
51
56
DAYS
P.H. = PARTIAL HEPATECTOMY
T.S. = TEST SUBSTANCE
B: PROMOTION ASSAY
DENA
SACRIFICE
PROMOTION REGIMEN
0
5
56
60-61
DAYS
DENA = DIETHYLNITROSAMINE
PROMOTION REGIMENS
A. TWICE WEEKLY DOSES OF TRIHALOMETHANE
B. 500 PPM SODIUM BARBITAL IN DRINKING WATER

FIGURE 1. Schematic presentation of the initiation and promotion protocols of the rat liver foci bioassay.

in tricaprylin (2 ml/kg body weight) or tricaprylin (2 ml/kg body weight) twice weekly for a total of 53 days. Other rats received 500 ppm sodium barbital in their drinking water for the same length of time (positive control). Four to five days after the termination of the promotion regimens, the rats were sacrificed. Their liver were excised and scored for GGTase-positive foci as described above.

Results

DNA Binding

The DNA isolated by the phenol extraction procedure, even though it contained less than 3% protein, required further purification in order to demonstrate that the radioactivity was bound to DNA and not to the contaminating protein. Cesium chloride isopycnic centrifugation was used to further purify the liver and kidney DNA from the rats treated with either bromoform-^{14}C (1.5 mmole/kg body weight, 2.7 mCi/mmole), chloroform-^{14}C (0.4 mmole/kg body weight, 15 mCi/mmole), or DMN-^{14}C (0.1 mmole/kg body weight, 50.9 mCi/mmole) and from the liver of mice treated with chloroform-^{14}C (1 mmole/kg body weight, 15 mCi/mmole). In rat liver and kidney, a definite peak of radioactivity derived from chloroform was found associated with the ultraviolet-absorbing peak containing the DNA, whereas no association was found for chloroform in mouse liver and bromoform in rat liver (Fig. 2). The binding index of chloroform to rat liver and kidney DNA was 0.017 and 0.0055, respectively, which represents 0.05–0.15% the binding index for DMN (11.4, Table 1).

The radioactivity present in kidney DNA from rats treated with chloroform-^{14}C was demonstrated to represent the formation of adducts in contrast to incorporation during *de novo* DNA synthesis (Fig. 3). The kidney DNA was hydrolyzed in 0.1*N* HCl and the hydrolyzate chromatographed on a HPLC equipped with a semipreparative Partisil 10 SCX ion exchange column. Over 95% of the applied radioactivity was eluted in the early peak containing the pyrimidines and deoxyribosyl phosphate backbone of the DNA. There was no evidence for the incorporation of radioactivity into adenine and guanine. Since over 60% of the radioactivity was previously demonstrated to be bound to the DNA (Fig. 2*C*), it was unlikely that the radioactivity in the early peak represented contaminating protein or incorporation by *de novo* synthesis of the DNA constituents with exclusion of the purines. Therefore, chloroform appeared to bind DNA at the pyrimidines, or phosphate and/or to form dinucleotide linkage.

Initiation Assay

Bromoform (0.8 mmole/kg body weight) and chloroform (1.5 mmole/kg body weight) were tested in the initiation protocol of the rat liver foci bioassay (Fig. 1). The trihalomethanes did not increase the incidence of GGTase-positive foci when administered to either intact or partial hepatectomized rats (Table 2). DENA (0.5 mmole/kg body weight), the positive control, resulted in the expected positive response. The initiating activity, if any, of trihalomethanes was much less than DENA.

Promotion Assay

The promoting activity of chloroform was determined in the promotion protocol of the rat liver foci bioassay (Fig. 1). Chloroform (1.5 mmole/kg body weight) administered twice weekly for 53 days to non-initiated rats or DENA administered to non-promoted rats did not result in a statistically significant increased incidence of GGTase-positive foci (Table 3). A slight but significant increase in the incidence of GGTase-positive foci compared to untreated controls was observed in rats that received the initiating dose of DENA followed by the chloroform promotion regimen. The incidence of GGTase-positive foci in animals initiated with DENA and promoted with chloroform was not significantly different from the incidence in animals treated either only with DENA or with chloroform.

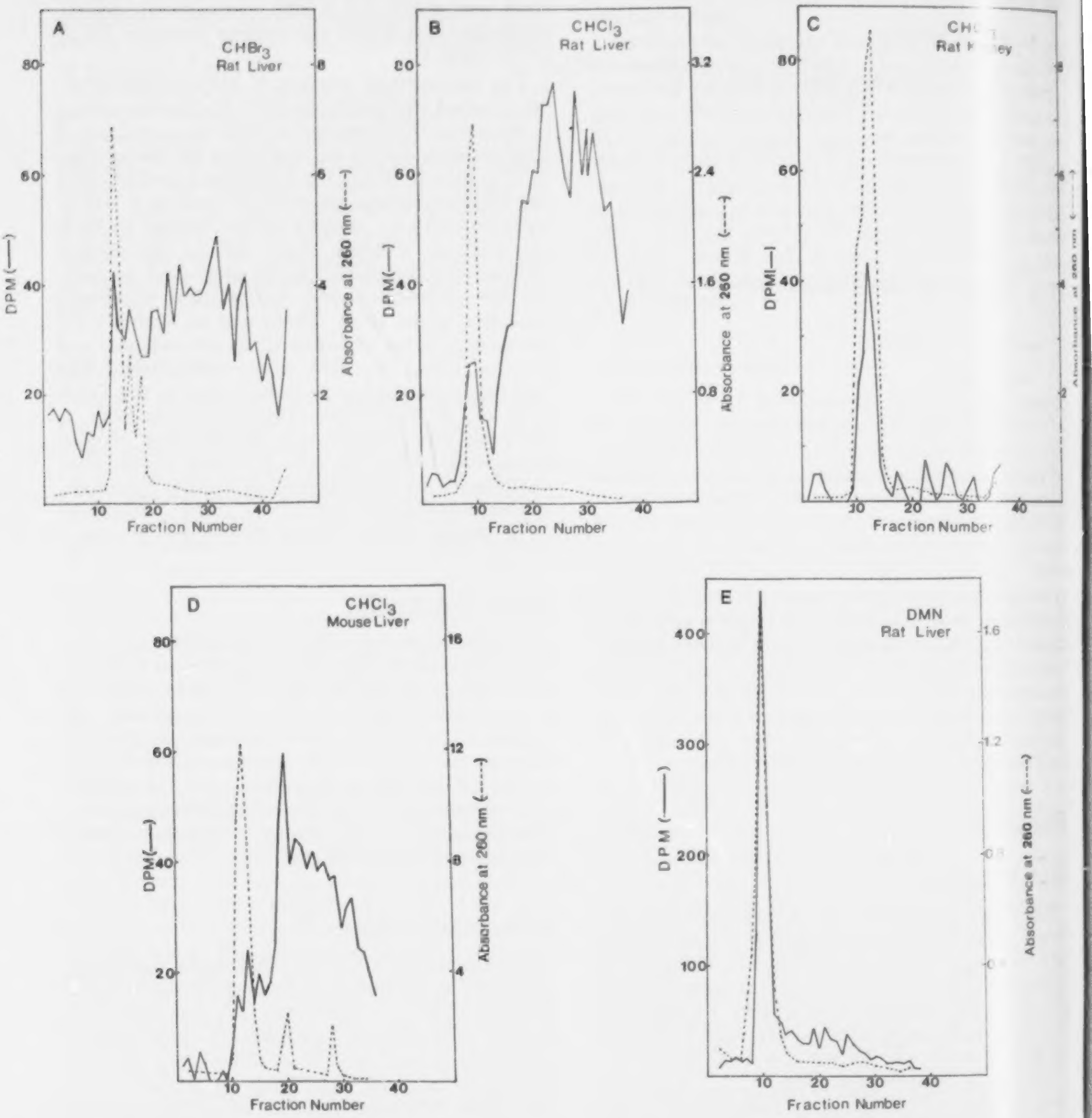

FIGURE 2. Cesium chloride isopyknic centrifugation of DNA. The DNA was dissolved in 0.25% SDS and layered on a CsCl solution (1.80 g/cm^3) so that the final density was 1.59 g/cm^3. Centrifugation was performed in a Beckman SW 50.1 rotor at 35,0000 rpm for 65 hr. Fractions were collected by puncturing the bottom of the centrifuge tube and were monitored for (———) radioactivity (dpm) (—) absorbance at 260 nm and density: (*A*) liver DNA from bromoform treated rats, amount applied 1.3 mg, and 1990 dpm; (*B*) liver DNA from chloroform-treated rats, amount applied 0.46 mg and 1475 dpm; (*C*) kidney DNA from chloroform-treated rats, amount applied 2.9 mg and 438 dpm; (*D*) liver DNA from chloroform-treated mice, amount applied 2.8 mg and 875 dpm; (*E*) liver DNA from dimethylnitrosamine-treated rats, amount applied 0.32 mg and 1890 dpm.

Table 1. Binding of trihalomethanes to DNA.

Chemical	Species	Organ	Binding index CsCl[a]
Chloroform	Rat	Liver	0.017
	Rat	Kidney	0.0055
	Mouse	Liver	< 0.0012
Bromoform	Rat	Liver	< 0.015
DMN[b]	Rat	Liver	11.4

[a] Binding index = pmole bound per mg DNA/μmole kg body weight.

[b] DMN = dimethylnitrosamine.

Discussion

Chloroform is an environmental carcinogen found in drinking water (*3,4*). Estimation of the human health hazard as a consequence of this exposure to chloroform requires extrapolation of the animal carcinogenicity data to humans. Models for the extrapolation of animal data to humans are being developed. Two possible mechanisms of action for the carcinogenicity of chloroform are genetic (initiation) and/or epigenetic (promotion). The determination of the contribution of these two mechanisms of action to the carcinogenicity of chloroform is required prior to the adaptation of the appropriate extrapolation model.

The possible initiating activity of chloroform was investigated by determination of the binding to DNA and the ability to initiate an increased incidence of GGTase-positive foci in the rat liver foci bioassay (*10-12*). Chloroform was demonstrated to bind rat liver and kidney DNA but there was no evidence for binding to mouse liver DNA within the sensitivity of the assay. The total binding of chloroform or bromoform to rate liver or kidney DNA was less than 0.15% the binding of DMN to rat liver DNA. The low level of DNA binding by bromoform and chloroform indicated that the contribution of the genetic or initiating component to the carcinogenicity of the trihalomethanes was much less than the genetic component of DMN. In the rat liver foci bioassay for initiating activity, bromoform and chloroform when administrated to either partial hepa-

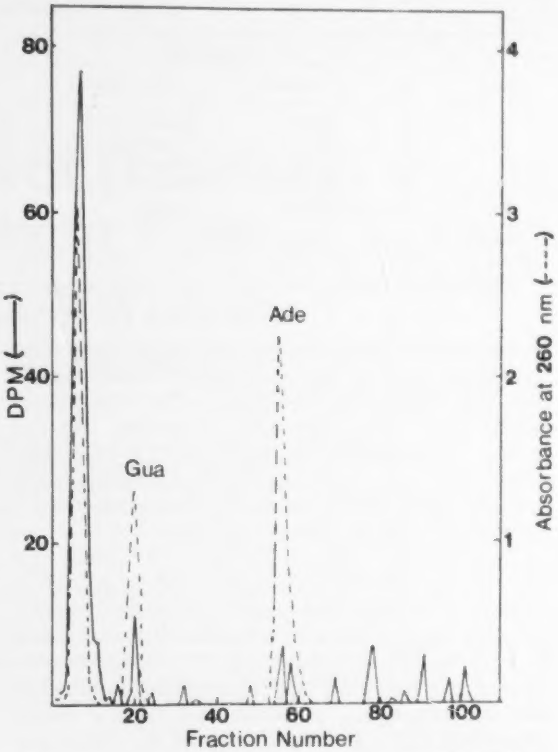

FIGURE 3. HPLC elution profile of acid-hydrolyzed DNA. Kidney DNA from chloroform treated rats was hydrolyzed in 0.1*N* HCl at 70°C for 30 min. The hydrolyzate was applied to a high performance liquid chromatograph equipped with a semipreparative Partisil 10 SCX ion exchange column. The column was eluted at a flow rate of 4 ml/min with 0.025*M* ammonium phosphate (pH 4.0) for 20 min, followed by a linear increase over 20 min in the concentration to 0.25*M* ammonium phosphate (pH 4.0). The eluate was monitored for absorbance at 260 nm and 2 ml fractions collected for determination of radioactivity.

Table 2. Initiation assay of trihalomethanes.

Group	*N*	Treatment			GGTase foci, foci/cm²
		Initiation	Partial hepatectomy	Promotion[a]	
A	12	Chloroform	+	+	1.08 ± 0.28[b]
B	12	Chloroform	−	+	0.40 ± 0.21
C	12	Bromoform	+	+	0.48 ± 0.12
D	12	Bromoform	−	+	0.26 ± 0.18
E	12	Tricaprylin	+	+	1.07 ± 0.24
F	12	Tricaprylin	−	+	0.13 ± 0.07
G	12	DENA	+	+	15.8 ± 1.9

[a] Promotion was accomplished with 500 ppm sodium phenobarbital in drinking water for 47 days.

[b] Results are means ± standard errors.

Table 3. Promotion assay of chloroform.

Group	N	Treatment		Weight, g[a]		Organ body weight × 100[a]	GGTase foci, foci/cm^2[a]
		Initiation	Promotion	Body	Liver		
A	15	Water	Tricaprylin	498 ± 12[b]	15.1 ± 0.5	3.0 ± 0.06	0.45 ± 0.14
B	16	DENA	Tricaprylin	497 ± 10	15.4 ± 0.5	3.0 ± 0.13	0.90 ± 0.31
C	15	Water	Chloroform	466 ± 8.6	15.0 ± 0.5	3.2 ± 0.05	1.42 ± 0.44
D	16	DENA	Chloroform	466 ± 9.4	14.6 ± 0.5	3.1 ± 0.08	2.22 ± 0.63[c]
E	10	DENA	Barbital	—	—	—	3.02 ± 1.10[c]

[a] Results are means ± standard errors.

[b] The initial body weights of the groups were 260-268.

[c] Different from group A by a nonparametric test with $p < 0.05$.

tectomized or intact rats and followed by promotion with phenobarbital did not initiate GGTase-positive foci. Initiation of GGTase-positive foci was demonstrated for DENA (the positive control). The low level of DNA binding and the failure to demonstrate initiating activity in the rat liver foci bioassay would indicate that any initiating activity of chloroform is substantially lower than nitrosamines.

Chloroform is toxic to the liver and kidney (*14, 15*) which are the target organs of carcinogenesis in rodents. The hepatic response to chloroform in rats included regenerative hyperplasia (*16*) and induction of ornithine carboxylase (*17*). Regenerative hyperplasia and induction of ornithine decarboxylase are properties possessed by hepatic tumor promoters (*18, 19*). We attempted to obtain evidence in the rat liver foci bioassay for the tumor promoting activity of chloroform. In rats initiated with DENA, subsequent treatment with chloroform increased the incidence of GGTase-positive foci when compared to untreated rats. However, when compared to rats that received only the DENA or chloroform the increased incidence of foci was not significant. Therefore, further confirmatory studies are required to demonstrate the tumor promoting activity of chloroform. In summary, chloroform would appear to possess (1) very little if any tumor-initiating activity and (2) as yet unproven tumor-promoting activity.

The authors sincerely appreciate the excellent technical help of Ms. M. J. Rouse.

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Environmental Health Perspectives
Vol. 46, pp. 157-162, 1982

Chloroform Induction of Ornithine Decarboxylase Activity in Rats

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Chloroform is a drinking water contaminant that has been demonstrated to be carcinogenic to mice and rats resulting in an increased incidence of liver and kidney tumors, respectively. The mechanism of chloroform carcinogenicity might be by tumor initiation and/or promotion. Since induction of ornithine decarboxylase (ODC) activity has been proposed as a molecular marker for tumor promoters, we have investigated the effect of chloroform on ODC activity in rats. Chloroform induced a dose-dependent increase of hepatic ODC with an apparent threshold at 100 mg/kg body weight. Female rats were two to four times more susceptible to chloroform. Upon daily dosing of chloroform for 7 days the liver became less susceptible, with the last dose of chloroform resulting in only 10% of the activity observed after a single dose. Nuclear RNA polymerase I activity was also induced by chloroform. Chloroform, rather than increasing the activity of renal ODC, resulted in a 35% reduction. The induction by chloroform of hepatic ODC activity might be associated with regenerative hyperplasia while the renal carcinogenicity of chloroform could not be demonstrated to be associated with ODC induction.

Introduction

Chloroform has been demonstrated in mice and rats to increase the incidence of hepatocellular carcinoma and epithelial tumors of the kidney, respectively (*1*). The initiation of carcinogenesis is believed to involve a somatic mutation; however, chloroform was nonmutagenic in the Ames Salmonella/microsome assay (*2*), in *E. coli* K 12 for basepair substitution (*3*), and in the 8-azaguanine locus in Chinese hamster lung fibroblast in cell culture (*4*). It also appeared that the amount of covalent binding of chloroform to rat liver and kidney DNA was very minimal (*5*). The negative evidence for chloroform mutagenicity and the low level of DNA interaction has resulted in the proposal that chloroform is not an initiator of carcinogenesis but is rather a tumor promoter.

Chloroform has long been known to be toxic in the liver and kidney, which are the organs in which an increased incidence of tumor formation has been found (*1,6,7*). The hepatotoxicity include fatty degeneration, glycogen depletion, vacuolation, swelling and necrosis. The severe tissue damage that resulted in regenerative growth of the liver (*8*) could stimulate the progression of preneoplastic cells to tumors. This proposed epigenetic stimulation of the neoplastic progression is called promotion.

Ornithine decarboxylase (ODC) induction has been associated with tumor promotion in skin (*9*) and liver (*10*), so that it might be a molecular marker for tumor promotion. ODC is the first and rate-limiting enzyme in polyamine biosynthesis (*11*) and has a very rapid turnover time of 10-20 min in liver (*12,13*). Drugs, hormones, and partial hepatoectomy which increased cellular proliferation have been shown to increase ODC activity (*14*). In this communication, we report the effect of chloroform on ODC activity in rat liver and kidney.

Materials and Methods

Materials

Fischer 344 albino rats, weighing 175-200 g, were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Adrenalectomized rats were also purchased from Charles River. Purina

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Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and distilled water were provided *ad libitium*.

DL-Ornithine 1-^{14}C and uridine triphosphate-^{3}H were purchased from New England Nuclear (Boston, Mass.). Chloroform (glass distilled and without preservative), carbon tetrachloride and benzene were obtained from Burdick and Jackson Laboratories (Muskengun, Mass.), and Aroclor-1254 was purchased from Analab, Inc. (North Haven, Conn.).

Subcellular Fractionation

Animals were sacrificed by decapitation. The livers or kidneys were removed, rinsed, blotted, weighed and homogenized in 1.0 volume (1 ml/g tissue) of ODC homogenizing buffer (0.1*M* NaH_2PO_4, 0.8*mM* pyridoxal-5-phosphate, 2.0*mM* EDTA; pH 7.5) at 0°C with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 10,000*g* for 10 min at 4°C. The supernatant fraction was decanted off and further centrifuged at 144,000*g* for 60 min at 4°C. The 144,000*g* supernatant fraction was removed with a Pasteur pipet introduced below the surface to avoid the turbid lipid layer on top. This cytosol fraction was used as the enzyme source for the ornithine decarboxylase assay.

Liver nuclei used in the RNA polymerase I assay were prepared by the isolation procedure developed by Muramatsu et al. (*15*) and described by Yu and Feigelson (*16*).

Enzyme Assays

All animals for the ornithine decarboxylase assay were sacrificed between 9 and 10 AM. Cytoplasmic ODC activity was measured by determination of the release of $^{14}CO_2$ from ornithine (1-^{14}C) essentially as described by Bethell and Pegg (*17*). The cytoplasmic fraction (100 µl) was incubated with 900 µl of ODC incubation medium containing 56*mM* NaH_2PO_4, 3.8*mM* dithiothreitol, 2.3*mM* EDTA (pH 6.5) and 1.0 µCi of DL-ornithine (1-^{14}C) (51.3 mCi/mmole). After incubation for 30 min at 37°C the reaction was stopped by the addition of 0.3 ml of 5*M* H_2SO_4, and the $^{14}CO_2$ released was trapped on filter paper discs saturated with 0.25 ml of hyamine hydroxide. The production of $^{14}CO_2$ was linear with respect to the amount of enzyme protein added to each incubation. The discs were removed, placed in liquid scintillation vials containing 15.0 ml ACS (Amersham) and 0.41 ml of 0.5*N* HCl, and the radioactivity determined in a Beckman LS 8100 liquid scintillation counter (Beckman Instrument Inc., Palo Alto, Calif.). The background activity, determined with acid denatured cytosol, was subtracted in each case. Protein was measured by use of the Bio-Rad protein assay kit (Bio Rad, Richmond, Calif.). Enzyme activity was expressed as CO_2 evolved/mg protein/30 min incubation.

RNA polymerase I activity was determined by using purified intact nuclei as the enzyme source as described by Roeder and Rutter (*18*). Isolated nuclei were incubated in a final volume of 1.0 ml containing 10 mg pyruvate kinase (400 units/mg protein), 23 µmole Tris-HCl (pH 7.9), 1.0 µmole $MnCl_2$, 4.0 µmole KCl, 3.0 µmole NaF, 2.0 µmole phosphoenol pyruvate, 0.8 µmole 2-mercaptoethanol, 0.3 µmole each GTP, CTP, ATP, 0.06 µmole unlabeled UTP, 0.002 µmole ^{3}H-UTP (20.3 Ci/µmole), and 50 µmole ammonium sulfate and with and without 1*mM* α-amanitin (*19*). Following incubation for 10 min at 30°C, the reaction was terminated by the addition of 4.0 ml of 10% TCA. The samples were centrifuged at 3,000 rpm for 10 min in a Beckman TJ-6 table top centrifuge (Beckman Instrument Inc., Palo Alto). The supernatant was discarded and the pellet washed two times with 3.0 ml of 5% TCA. The pellet was resuspended in 3.0 ml of 5% TCA and collected under vacuum onto HA 0.45*mM* Millipore filters. The filters were washed with 10 ml of 5% TCA, followed by 5 ml of 60% ethanol and allowed to air-dry overnight. The next day they were oxidized in a Packard Model 306 sample oxidizer (Packard Instrument Co., Downers Grove, Ill.). Radioactivity was determined by liquid scintillation counting and protein measured as described above. The data were expressed as pica moles of ^{3}H-UTP incorporated/10 min/mg protein.

Results

The effect of various carcinogens and tumor promoters on ornithine decarboxylase (ODC) activity in male rat liver was determined (Table 1). With the exception of benzene and saccharin, all of the compounds tested stimulated ODC. Chlorinated carcinogens and tumor promoters such as chloroform, carbon tetrachloride and the polychlorinated biphenyl, Aroclor-1254, caused the greatest increase in ODC activity.

Chloroform Time Course and Dose Response

The time course of chloroform induction of ODC in male rat liver was determined (Fig. 1). Enhancement was evident as early as 2 hr and slowly increased until 18 hr, at which time maximum enhancement of ODC activity occurred. The maximum enhancement of ODC activity was 43.5-fold control level and 74 pmole CO_2/30 min/mg protein. By 24 hr, the peak enhancement of ODC activity had decreased by 80%.

Table 1. Effect of suspected tumor promoters on rat hepatic ornithine decarboxylase activity.

Compound[a]	Dose, mg/kg	ODC activity[b]	Enhancement[c]
Aroclor-12	800	38.1 ± 4.93[d]	22.4
Benzene	440	2.6 ± 0.69	1.5
Carbon tetrachloride	800	25.0 ± 4.30[d]	14.7
Chloroform	750	74.0 ± 11.6[d]	43.5
Methapyrilene	10	9.3 ± 2.96[d]	5.5
Phenobarbital	100	9.6 ± 1.00[d]	5.7
Saccharin	1000	2.3 ± 0.31	1.4
Testosterone	300	13.2 ± 4.00[d]	7.8

[a] Animals were injected intraperitoneally with the compound and sacrificed 18 hr later at 9:00 AM.
[b] ODC activity was determined by measuring the release of $^{14}CO_2$ from DL-ornithane (1–^{14}C) and was expressed as pmole CO_2/30 min incubation/mg protein. Results are means from eight animals ± standard error.
[c] Enhancement of ODC activity compared to the mean of eight saline-treated rats which was 1.7 ± 0.44.
[d] Statistically greater than the activity in saline-treated rats by the Student t-test at the level of $p < 0.01$.

The administration of a daily dose of chloroform for 7 days resulted in a lower enhancement of ODC activity compared to a single dose (Fig. 2). After seven total doses of chloroform, the ODC activity was only 10% the activity observed after the first dose. The decreased responsiveness of the liver to chloroform was even apparent for a second dose of chloroform administered on the following day.

The dose-response relationship of ODC induction by chloroform in female and male rats was performed (Fig. 3). Female rats were two to four times as sensitive to chloroform as male rats. There was an apparent threshold in male rats for ODC induction below 100 mg/kg body weight. The ODC activity increased with dose of chloroform until 750 mg/kg body weight in both female and male rats. Higher doses of chloroform could not be tested in females due to a high incidence of lethality.

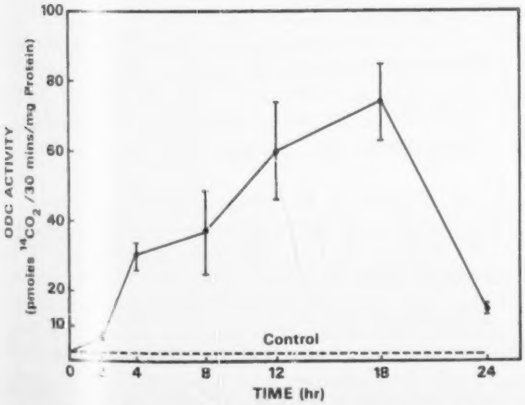

FIGURE Time course of chloroform induction of ODC in male rats. Male rats were injected intraperitoneally with 750 mg/kg and sacrificed after various intervals of time at 9:00 AM. All points represent the mean of a minimum of eight animals ± S.E.

Renal ODC Activity

The ODC activity of male rat kidney was approximately 300-fold the activity in male rat liver (Table 2). The function of this high level of ODC activity in male kidney is unknown; however, after unilateral nephrectomy, a fourfold increase of ODC activity occurred 24 hr later in the regenerating kidney

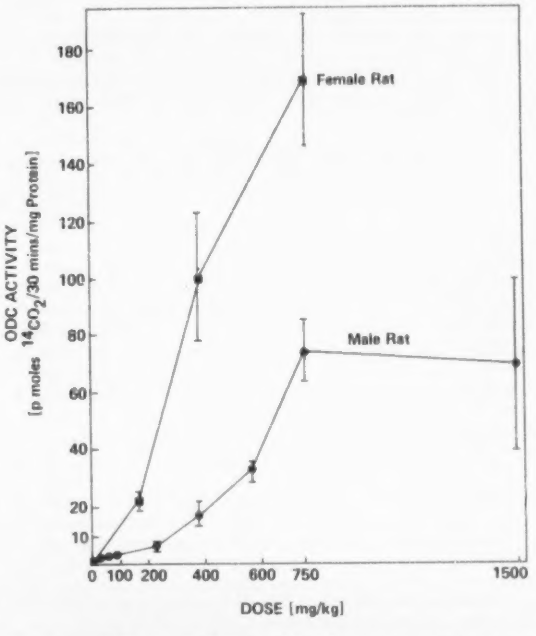

FIGURE 2. Effects of chronic chloroform treatment on rat hepatic ornithine decarboxylase activity. Male rats were injected intraperitoneally with 500 mg/kg $CHCl_3$ daily at 3:00 PM for a period of up to 7 days. Rats were sacrificed 18 hr after their last dose. All points represent the mean of a minimum of eight animals ± SE.

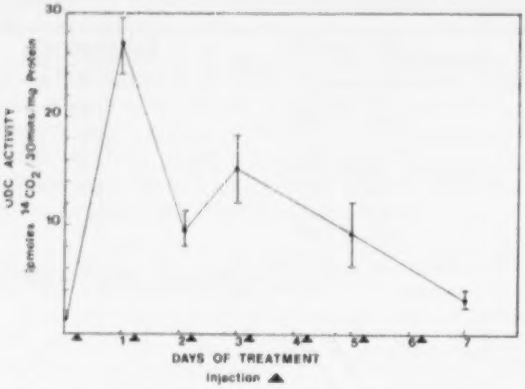

FIGURE 3. Dose-response relationship of chloroform induction of hepatic ODC. Rats were injected intraperitoneally with the appropriate dose of $CHCl_3$ and sacrificed 18 hr later at 9:00 AM. The ODC activity in saline-treated control female and male rats was 1.7 ± 0.44 and 1.55 ± 0.38 enzyme units, respectively. All points represent the mean of a minimum of eight animals ± SE.

Table 2. Effect of $CHCl_3$ on rat renal ODC activity.

Compound	Dose, mg/kg[a]	Time, hr	ODC activity[b]
Saline	—	18	482.23 ± 24.23
$CHCl_3$	750	18	317.65 ± 33.55

[a] Animals were injected intraperitoneally with either saline or $CHCl_3$ and the animals sacrificed 18 hr later at 9:00 AM.

[b] ODC activity was expressed as pmole CO_2 liberated/30 min/mg protein. Results are means of eight animals ± standard errors.

(*20*). Chloroform (750 mg/kg body weight), which resulted in a 43.5-fold enhancement of rat liver ODC activity (Table 1), caused a 35% reduction of kidney ODC activity (Table 2).

Hormonal Modulation of Chloroform-Induced ODC

Adrenalectomy prevented the increase in rat liver ODC activity that resulted from treatment with barbiturates (Table 3). The extent of chloroform induction of ODC activity was not statistically diminished by prior adrenalectomy. Therefore, unlike barbiturates, the enhancement of rat liver ODC by chloroform was not dependent on the adrenal gland. Pretreatment with propranolol (a beta adrenergic receptor blocker) or 17-α-hydroxyprogesterone (a corticosterone receptor blocker) also did not diminish the increased level of ODC induced by chloroform.

RNA Polymerase I Response to Chloroform

RNA polymerase I is another enzyme that has been demonstrated to increase during the hyperplastic response in rat liver (*14*). Chloroform induction of RNA polymerase I activity was determined

Table 3. Effects of hormonal manipulations on chloroform-induced hepatic ornithine decarboxylase.

Compound	Dose, mg/kg[a]	Surgery[b]	ODC activity
Saline	—	—	1.7 ± 0.44
Saline	—	Adrenalectomy	3.2 ± 0.63
$CHCl_3$	187.5	—	9.5 ± 5.00
$CHCl_3$	187.5	Adrenalectomy	13.5 ± 5.58
$CHCl_3$	375.0	—	17.7 ± 4.71
$CHCl_3$	375.0	Adrenalectomy	12.6 ± 3.16
$CHCl_3$	750	—	74.0 ± 11.6
$CHCl_3$	750.0	Adrenalectomy	47.5 ± 19.5
17-Hydroxyprogesterone (HP)	20	—	1.3 ± 0.36
17-HP + $CHCl_3$[e]		—	110.1 ± 13.7
Proproanolol	10	—	1.7 ± 0.38
Propranolol + $CHCl_3$[f]		—	76.9 ± 15.6
Barbital	200	—	13.1 ± 3.00
Barbital	200	Adrenalectomy	1.1 ± 0.52
Phenobarbital	100	—	9.6 ± 1.00
Phenobarbital	100	Adrenalectomy	2.6 ± 0.89

[a] Animals were injected intraperitoneally and sacrificed 18 hr later. The 17α-hydroxyprogesterone and propranolol were injected 30 min prior to the chloroform and sacrificed 18 hr later.

[b] Adrenalectomy was performed by Charles River laboratory. The rats were maintained on saline for their drinking water and used 14 days after surgery.

[c] ODC activity was expressed as pmole CO_2/30 min incubation/mg protein.

[d] Results are means of eight animals ± standard errors.

[e] The dose of 17α-hydroxyprogesterone was 20 mg/kg body weight and of chloroform 750 mg/kg body weight.

[f] The dose of propranolol was 10 mg/kg body weight and of chlorofrom 750 mg/kg body weight.

[g] Statistically decreased from intact animal by the Student *t*-test at the level of significance of $p < 0.01$.

Table 4. Effect of $CHCl_3$ on rat hepatic RNA polymerase I.

Compound	Dose, mg/kg[a]	Time, hr[b]	RNA polymerase I activity[c]
Saline	—	18.5	138.03 ± 16.02[d]
$CHCl_3$	750	18.5	319.40 ± 36.14

[a] Animals were injected intraperitoneally with either saline (1 mg/kg body weight) or $CHCl_3$.

[b] The animals were sacrified 18.5 hr after treatment at 9:00 a.m.

[c] RNA polymerase I activity was calculated in the presence of 1*mM* α-amanitin and was expressed as pmole UTP incorporated/10 min/mg protein.

[d] Results are means of eight animals ± standard errors.

in the presence of 1*mM* α-amanitin (Table 4). The α-amanitin was used to inhibit RNA polymerase II and III (Table 4). Chloroform compared to saline treated rats resulted in a 2.3-fold enhancement of liver nuclear RNA polymerase I activity.

Discussion

The induction of hepatic ODC has been demonstrated to be associated with the hyperplastic and regenerative response to drugs, hormones and partial hepatectomy (*21*). Chloroform has been demonstrated to induce a regenerative hyperplasia in rat liver (*8*). Chloroform caused a dose-dependent enhancement of hepatic ODC that could be associated with the regenerative hyperplasia. An apparent threshold for chloroform induction of ODC activity was indicated below 100 mg/kg body weight which might indicate a threshold for regenerative hyperplasia. Female rats compared to males exhibited a 2- to 4-fold greater response of ODC induction. Chloroform also induced hepatic nuclear RNA polymerase I, another enzyme that has been associated with the hyperplastic response in liver (*14*). The induction of ODC and hyperplasia has been proposed as molecular markers for tumor promoters (*9*).

Tumor promotion appears to require the prolonged and repeated administration of the promoter (*9*). Upon daily administration of chloroform, the extent of the enhancement decreased so that after a total of seven doses, the ODC activity was only 10% the level after a single dose. This reduction in the effect of chloroform could have resulted from the inhibition of accumulated putrescine (*22*). The hyperplastic response of α-hexachlorobenzene in the liver as measured by the increase in DNA replication, was resistant to a second dose (*23*). The hyperplastic response of chloroform as indicated by the reduced enhancement of ODC might also be limited.

The kidney was the target organ of chloroform carcinogenicity in rats. Kidney ODC activity was not induced by chloroform but was rather reduced. The level of ODC activity in kidneys was approximately 300-fold the hepatic level. Even in presence of this high control level, unilateral nephrectomy was capable of inducing ODC in the regenerating kidney (*24*). Therefore, the carcinogenicity and possible tumor promoting activity of chloroform in rat kidney would appear not to be associated with the induction of ODC. In summary, the hepatic regenerative hyperplasia induced by chloroform in rats appeared to be associated with the induction of ODC while the renal carcinogenicity was not associated.

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Environmental Health Perspectives
Vol. 46, pp. 163-168, 1982

Mechanistic Considerations for Carcinogenic Risk Estimation: Chloroform

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Chloroform has been reported to induce cancer in rodents after chronic administration of high doses by gavage. However, the interpretation of these findings is hampered by a lack of knowledge concerning the relative roles of genetic and nongenetic mechanisms in these bioassays. The present studies were carried out in male B6C3F1 mice in order to investigate the potential of chloroform to induce genetic damage and/or organ toxicity at the sites where tumors have been observed in the various bioassays. These studies revealed that carcinogenic doses of chloroform produced severe necrosis at the sites where tumors later developed. This was demonstrated by light microscopy as well as by determination of the cellular regeneration index following administration of ^{3}H-thymidine. Noncarcinogenic doses of chloroform failed to induce these responses. In contrast, studies of DNA alkylation and DNA repair *in vivo* failed to give any indication that chloroform had produced the type of genetic alterations associated with known genotoxic chemicals. These data suggest that the primary mechanism of chloroform-induced carcinogenesis is nongenetic in nature. If the same mechanism predominates in man, there should be little to no carcinogenic risk associated with exposure to noncytotoxic levels of chloroform.

Introduction

Some chemical carcinogens apparently act through direct alteration of DNA (by the induction of somatic mutations). This has led to the speculation that a single molecular event might influence the rate of cancer formation. Such a concept does not allow for the existence of an absolute threshold in carcinogens acting by this mechanism. However, considerations such as the multistage nature of chemical carcinogenesis, the existence of DNA repair systems and immune surveilance mechanisms, and the observation of threshold doses for other pathological responses support a possible threshold for at least some carcinogenic agents (*1*). There has been, and continues to be, considerable debate on the subject of thresholds in carcinogenesis.

Consequently, there has been some concern whenever large numbers of people are exposed to a material which has been shown to be an animal carcinogen, even if the levels of exposure are very much lower than those employed for the animal test. Chloroform is one example of such a chemical. It was tested for possible carcinogenicity following chronic administration by gavage (*2*) and found to induce liver tumors in B6C3F1 mice, as well as kidney and thyroid tumors in Osborne-Mendel rats.

Human exposures to chloroform are very much lower than those employed in the gavage bioassay. The primary source of exposure is to small amounts of trihalomethanes (including chloroform) formed during the chlorination of drinking water supplies. The Environmental Protection Agency has recently established a maximum contaminant level (MCL) for chloroform in finished drinking water of 100 ppb (0.1 mg.). The level chosen by EPA is based on a risk estimation which suggests that the excess cancer risk in populations exposed to this level of chloroform may be as high as 100 cases per million exposed population (*3,4*).

The risk estimation cited by the EPA is based upon the techniques outlined by the National Academy of Sciences (NAS) (*5*) in their book, "Drinking Water and Health." However, there are at least two reasons why these techniques may not be appropriate for risk extrapolations with chloroform: (1) The NAS indicated that they felt it was "prudent" to assume, when there was no evidence to indicate otherwise, that animal carcinogens were acting through genetic mechanisms and hence could not be assumed to have any "threshold" in their activity. This assumption led them to recommend that mathematical models such as the "one-hit" model be considered for risk extrapolation. (2) The NAS also noted that, as a general rule, processes such as metabolism and excretion occur less rapidly in man than in laboratory rodents (*6*). Since these processes are often involved in detoxification of foreign chemicals, the NAS also suggested that, when there was no evidence to indicate otherwise, man should be considered to be more sensitive to the toxicity (including carcinogenicity) of foreign chemicals than rodents.

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However, there is reason to believe that neither of these assumptions are justified when the carcinogenic risk associated with exposure to low levels of chloroform is estimated. Pertinent data for a more realistic estimation of carcinogenic risk will be discussed below.

Methods

Animals

Male mice (CD-1 or B6C3F1 strains) and male rats (Sprague-Dawley strain) were obtained from Charles River Laboratories, Wilmington, Mass. All animals were acclimated for at least one week before use.

Materials

^{14}C-Chloroform was obtained from New England Nuclear (Catalog No. NEC-351) with a reported specific activity of 5.4 mCi/mmole and a radiochemical purity of > 97%. Nonradioactive chloroform was Baker Analytical Reagent grade containing 0.7% ethanol as a preservative (and less than 0.1% of any other materials). All other materials were reagent grade from commercial supply houses.

Enzymes used in the purification of DNA α-amylase, typsin, chymotrypsin, ribonuclease-A, and deoxyribonuclease (DN-100) were obtained from Sigma Chemical Co., St. Louis, Mo.

DNA Alkylation

The potential of chloroform to cause DNA alkylation *in vivo* was estimated by measuring the specific radioactivity of DNA isolated from animals sacrificed 4 hr after exposure to ^{14}C-chloroform (240 mg/kg, PO). DNA was isolated from the livers and kidneys of these animals by the method of Marmur (*7*) as modified by Reitz et al. (*8*).

The modified procedure involved the precipitation of isolated DNA from solution with 5% trichloroacetic acid (TCA), hydrolysis of the precipitate in a buffered solution of purified deoxyribonuclease, and reprecipitation of insoluble impurities by addition of more TCA (final concentration 5%). Following the second precipitation, the incubation is filtered through a Millipore filter (5 µm pore size) and the filtrate is collected. This last step removes many contaminating macromolecules such as protein, RNA, and glycogen.

These conditions are similar to those employed by others (*9–11*). The mild acid and enzyme treatments would not be expected to degrade covalently bonded DNA adducts, and levels of DNA alkylation obtained using these methods are consistent with those reported by other investigators (*12*).

DNA Repair *in Vivo*

DNA repair was estimated by administering nonradioactive chloroform to animals and subsequently determining the rate of incorporation of ^{3}H-thymidine into DNA in animals receiving doses of hydroxyurea sufficient to depress normal DNA synthesis. Details of this procedure have been described previously (*8*).

Other Analyses

Glycogen, RNA, and protein in the final DNA preparation were determined by the methods of Shields and Burnett (*13*), Brown (*14*), and Bradford (*15*), respectively. Radioactivity was determined by liquid scintillation counting with automatic quench correction (Beckmann LS-9000). DNA concentration was estimated by the method of Burton (*16*).

Cellular Regeneration

Male mice were gavaged with various doses of unlabeled chloroform, held for 4 hr, and then injected IP with ^{3}H-thymidine. Four hours later, these animals were sacrificed, and DNA was isolated from liver and kidney samples as outlined by Reitz et al. (*8*). The cellular regeneration index was estimated by determining the relative specific radioactivity in the DNA from treated and control groups.

Histopathological Assessment

Samples of kidney and liver were removed at necropsy and fixed in buffered 10% formalin. The tissues were processed by routine histologic procedures. Sections (5–6 μm) were stained with hematoxylin and eosin and examined by light microscopy.

Statistical Analyses

Data were analyzed by the procedure of Wilcoxon (*17*) as modified by Mann and Whitney (*18*) for unequal sample sizes. Outliers were removed as outlined by Grubb (*19*) before analysis. The level of significance was chosen to be $p < 0.05$.

Results

DNA Alkylation

The levels of radioactivity incorporated into DNA isolated from the liver and kidney of B6C3F1 mice exposed to ^{14}C-chloroform are summarized in Table 1. These data are reported as micromole equivalents of chloroform bound per mole of DNA phosphorus, adjusted to an equivalent dose of 1 mmole/kg.

The levels of radioactivity in the DNA isolated from the organs of the chloroform-treated mice represented about 10 to 20 dpm over background counts in samples of DNA isolated from untreated mice. (All samples were counted for 100 min in order to accumulate sufficient counts for accurate estimation of the low amounts of radioactivity present.) It must be emphasized that the radioactivity observed in the DNA may also arise from biosynthetic incorporation of radioactive one carbon fragments during normal DNA synthesis. Hence the value reported must be considered an "upper limit" rather than an accurate estimate of the level of DNA alkylation.

Table 1. **Chemical binding indexes (CBI) for binding of various chemicals to DNA *in vivo* at a standard dose of 1 mmole/kg according to Lutz (*26*).**

Potency rating	Chemical, μmole/mole DNA
Strong	
Aflatoxin	17,000[a]
Dimethylnitrosamine	6,000[a]
Dimethylnitrosamine	7,430[b]
Moderate	
2-Acetylaminofluorene	560[a]
O-Aminoazotoluene	230[a]
Weak	
Urethane	29–90[a]
4-Dimethylaminoazobenzene	6[a]
Experimental results for selected chemicals	
Chloroform	1.5 (Det. Limit = 1)[b]
Perchloroethylene	0.0 (Det. Limit = 10)[b]

[a] Data from Lutz (*26*).

[b] Data gathered in Dow Laboratories.

DNA Repair

Repair of DNA (estimated as hydroxyurea-resistant incorporation of ^{3}H-thymidine into DNA) also served as an indicator of the potential of chloroform to cause genetic effects. Intraperitoneal administration of dimethylnitrosamine (DMN) caused large increases in DNA repair in the liver of B6C3F1 mice (Fig. 1), but chloroform (240 mg/kg, PO) was inactive in this system. Thus these data also fail to indicate any significant genotoxic activity for orally administered chloroform in mice.

Cellular Regeneration

Cellular regeneration was increased 14-fold in the liver of male mice treated with 240 mg/kg (Table 2). Much smaller effects on cellular regeneration in

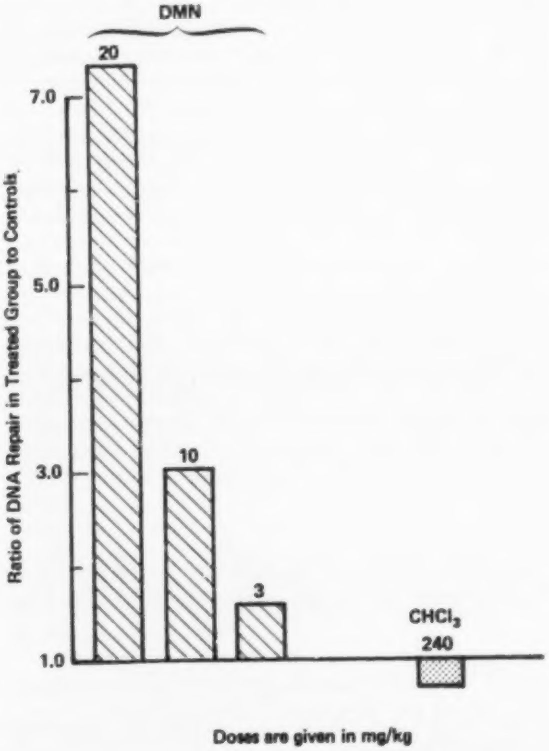

FIGURE 1. DNA repair in the liver of mice treated with dimethylnitrosamine (DMN) or chloroform ($CHCl_3$) relative to control groups.

Table 2. Cellular regeneration (estimated by determination of relative incorporation of ^{3}H-thymidine into DNA) in tissues of male B6C3F1 mice or male Osborne-Mendel rats 48 hr after a single gavage dose of chloroform.

Tissue/dose	DPM/g DNA ± SD	Ratio
Liver (mice)		
Control	3.6 ± 1.5 (n = 5)	—
15 mg/kg	3.4 ± 2.2 (n = 5)	0.94
60 mg/kg	8.0 ± 3.3 (n = 6)	2.2
240 mg/kg	50.6 ± 13.0 (n = 6)	14.0[a]
Kidney (mice)		
Control	2.4 ± 0.21 (n = 5)	—
15 mg/kg	1.9 ± 0.32 (n = 5)	0.79
60 mg/kg	19.6 ± 16.2 (n = 6)	8.2[a]
240 mg/kg	59.4 ± 10.6 (n = 6)	24.8[a]
Liver (rats)		
180 mg/kg		2.6[b]
Kidney (rats)		
180 mg/kg		1.4[b]

[a] Significantly different from control ($p < 0.05$, Mann Whitney U test).

[b] Data from Reitz et al. (*20*).

the liver were observed in the groups receiving either 60 mg/kg (2.2-fold increase) or 15 mg/kg.

The kidney tissue of mice was more sensitive than the liver tissue to the effects of chloroform. Cellular regeneration was increased 25-fold in the kidneys of mice receiving 240 mg/kg, and 8-fold in mice receiving 60 mg/kg. No effects on cellular regeneration were seen in the kidneys of mice receiving 15 mg/kg of chloroform by gavage (Table 2).

In order to further characterize the effects of chloroform on liver and kidney tissue, small groups of mice (two/dose) were gavaged with various doses of chloroform and then sacrificed 48 hr later for examination by histopathological techniques. Tissue damage was readily observable in the tissues where cellular regeneration had been stimulated (Table 3). Damage was severe enough to be judged necrotic following 240 mg/kg in both liver and kidney, while tubular epithelial regeneration was noted in the corticomedulary region of kidneys from mice gavaged with 60 mg/kg. No microscopic changes were observed in liver or kidney tissues obtained from mice gavaged with 15 mg/kg of chloroform (Table 3).

Previous experiments indicated that rats were less sensitive than mice to the toxic effects of chloroform, including the production of increased cellular regeneration. Male Osborne-Mendel rats dosed with 180 mg/kg of chloroform showed a 160% increase in cellular regeneration in the liver, and a 40% increase in the kidney (*20*) (Table 2).

Discussion

Chloroform has been tested in several of the standard tests designed to detect mutagenic (and hence presumably carcinogenic) potential. The results have been generally negative. For instance, two groups reported that chloroform failed to induce mutations in *Salmonella typhimurium* (*21*,*22*), and chloroform was also negative in a sex-linked recessive lethal test conducted in *Drosophila melanogaster* (*23*). Most recently, chloroform was selected as one of several prototype chemicals to be evaluated in a comprehensive battery of short term tests for carcinogenicity. The results of these tests were summarized by Bridges et al. (*24*) and Brooks and Preston (*25*). Chloroform was consistently negative in tests for genotoxicity (*24*, *25*).

The results of these studies of DNA alkylation and DNA repair are also inconsistent with the theory that chloroform can induce somatic mutations of the type that may lead to cancer. Doses of chloroform which exceeded those known to produce liver cancer in mice produced orders of magnitude less DNA alkylation than that observed with known genotoxic agents (Table 1). For comparative purposes, the degrees of alkylation of DNA produced in the target organs by known genotoxic chemicals (utilizing the same routes of administration known

Table 3. Histopathology in tissues of male B6C3F1 mice 48 hr after exposure to single oral doses of chloroform.

	No. mice affected/no. mice examined after various doses of chloroform			
Microscopic observation	0	15 mg/kg	0 mg/kg	240 mg/kg
Liver				
Individual hepatocellular necrosis; inflammatory cell infiltration	0/2	0/2	0/2	2/2
Increased mitosis	0/2	0/2	0/2	2/2
Centrilobular hepatocellular swelling (2/3 lobule involved)	0/2	0/2	0/2	2/2
Kidney				
No microscopic change	2/2	2/2	0/2	0/2
Severe diffuse renal cortical necrosis	0/2	0/2	0/2	2/2
Focal tubular epithelial regeneration; corticomedullary junction	0/2	0/2	2/2	2/2

to produce tumors) are summarized in Table 1. These data are from the publication of Lutz (*26*), and are all reported as micromoles of chemical bound per mole of DNA at a standard dose of 1 mmole/kg. Lutz observed a reasonable correlation between the *in vivo* potency for DNA alkylation and the potency as carcinogens (*26*) and proposed this method as a useful tool for estimating relative carcinogenic hazard.

Although it is not clear which sites of DNA are most sensitive to potentially mutagenic effects, or whether the various sites are equally susceptible to *in vivo* DNA repair mechanisms, the very low aklylation of DNA observed after chloroform administration suggests that the genotoxic potential of chloroform is minimal.

Furthermore, the failure of chloroform to stimulate any detectable activity in the DNA repair systems (Fig. 1) also suggests that no biologically significant alteration of DNA has occurred, even following administration of a dose which was carcinogenic in the NCI bioassay.

In contrast, severe tissue damage was produced in mice in the organs where tumors were reported to develop in two bioassays of chloroform (*2,27*). This damage was clearly visible at necropsy in the kidneys and livers of mice treated with high doses of chloroform.

This damage was also sufficient to stimulate cellular regeneration at these sites (Table 2). Both cellular regeneration and tissue damage were well correlated with the tumorigenicity in mice. For example, doses of 60 and 240 mg/kg stimulated cellular generation in the kidney, and Roe et al. (*27*) reported that 60 mg/kg induced kidney tumors in one of four strains of male mice. 15 mg/kg of chloroform failed to induce cellular regeneration and also was inactive (at any site) in the bioassay of Roe et al. The liver of male mice was less sensitive to the administration of chloroform. Cellular regeneration was not stimulated greatly at doses of chloroform below 240 mg/kg (Table 2), and liver tumors were only observed in mice after administration of doses above 100 mg/kg (*2*).

The presence of toxicity sufficient to produce necrosis (with attendant cellular regeneration) throughout the lifetime of the animal is a very significant observation, because it is well known that rapidly dividing cells are more sensitive to mutagenic stimuli than are quiescent cells (*28,29*). Increased cellular regeneration has been correlated with the tumorigenicity of other materials such as vinylidene chloride (*8*) and perchloroethylene (*30*). Increased cellular regeneration apparently preceded tumor development in every case in the bioassays of chloroform in mice.

The role of cellular regeneration in the chloroform bioassays in rats is not as clear. The NCI did not observe treatment-related tumors of the liver in Osborne-Mendel rats, and this is consistent with a much lower stimulation of cellular regeneration in the liver of rats following gavage with chloroform (*20*) as well as the report of Bull et al. (*31*) that the fatty infiltration of liver observed in B6C3F1 mice consuming drinking water with high levels of chloroform was not detected in Osborne-Mendel rats under similar conditions.

However, cellular regeneration was not greatly stimulated in the kidney of rats following acute treatment with 185 mg/kg chloroform (*20*), and this is a site where tumors did develop in the NCI bioassay. Clearly the effects of chronic chloroform treatment on the kidney of rats need to be better characterized.

Nevertheless, mice appear to be clearly more sensitive to the toxic effects of chloroform, including carcinogenicity, than rats. As discussed elsewhere (*32*), the toxicity of chloroform is apparently due to the production of a reactive metabolite produced by mixed function oxidases from the relatively inert chloroform molecule. Chloroform thus appears to belong to a class of chemicals which require metabolic activation for toxicity. Consequently, any species variation in the capacity to metabolize chloroform should dramatically affect the oncogenicity of this compound.

Mice have a much higher rate of oxidation of halogenated hydrocarbons to reactive intermediates than rats, and most of the halogenated hydrocarbons which produce tumors are more active in mice than rats. Since the relative activity of similar enzymes in man is less than either of the two rodent species (*5,6*) these considerations suggest that man should be less sensitive than either rodent species to any tumorigenic activity of chloroform.

Chang and Periera (*33*) have studied the alkylation of hemoglobin by ^{14}C-chloroform in rats and mice, and they found that, in contrast to cytotoxicity and carcinogenicity, the levels of alkylation were very similar in the two species. The reason for this discrepancy is not clear, but it may be that the rate of bioactivation (relative to the rate of detoxification through some pathway such as glutathione conjugation) is more critical than the total amount of metabolite formed.

In summary, chloroform failed to demonstrate genotoxic potential in the following battery of tests: bacterial mutagenicity; mammalian cell mutagenicity; dominant lethal tests in *D. melanogaster*; DNA alkylation *in vivo*; DNA repair *in vivo*.

However, the carcinogenicity of chloroform, particularly in mice, did appear to be correlated with the production of recurrent cytotoxicity. When such cytotoxicity is produced through the lifetime of an animal, increased frequencies and/or shorter latencies of spontaneous tumors may be expected.

These considerations provide a firm basis for discarding procedures such as the "one-hit" model for risk estimation, since these assume a genotoxic component. Increased tumor frequencies would not be expected from exposure to levels of chloroform below the cytotoxic threshold, since the genotoxic potential of chloroform appears to be nil.

This work was presented orally in part at the 3rd Conference on Water Chlorination, Colorado Springs, Colo., November 1, 1979. In addition, a portion of this material was presented orally to the Toxicology Forum, Aspen, Colo., July 26, 1980.

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Environmental Health Perspectives
Vol. 46, pp. 169-177, 1982

Case-Control Cancer Mortality Study and Chlorination of Drinking Water in Louisiana

by Marise S. Gottlieb* and Jean K. Carr*

Several Louisiana parishes (counties) using the Mississippi River for their source of public drinking water have the highest mortality rates (1950-69) in the United States for several cancers. Therefore, a case-control mortality study on cancer of the liver, brain, pancreas, bladder, kidney, prostate, rectum, colon, esophagus, stomach, non-Hodgkin's lymphoma, multiple myeloma, leukemia, Hodgkin's disease, lung, breast and malignant melanoma, from 1960 to 1975 in South Louisiana parishes grouped for similarities in industrial characteristics, having approximately equal exposure of the population to surface and groundwater, was conducted. Noncancer deaths were randomly selected as controls and matched to the case death on age, race, sex, and year and parish group of death. Water source at death was assigned based on the residence at death and described as surface or ground and chlorinated or nonchlorinated.

A significantly increased risk for surface, chlorinated water use was noted for rectal cancer. No risk could be demonstrated for colon cancer. The risk noted for bladder cancer by other investigators is not substantiated. Brain cancer risk appears to be associated with chlorinated groundwater, but this may be industrial confounding. Breast cancer demonstrated a slight, but significant, risk associated with surface chlorinated water. This risk, however, might be due to confounding of rural life style, early childbearing and large families with nonchlorinated water found in these settings. Chlorination risk for kidney cancer was not significant. No risk was observed in association with surface water for other cancers of the gastrointestinal or urinary tract. Multiple myeloma was significantly associated with a risk from ground water.

Introduction

Because South Louisiana is an area where comparatively large amounts of organic contaminants have been detected in municipal water supplies and because it is also an area with extremely high mortality rates for cancers of several sites and for all sites combined in selected counties (parishes) which have different sources of drinking water, it was a natural location to investigate what relationship, if any, exists between water quality and cancer incidence (*1-4*). Beginning in 1974 with the draft report released by the Environmental Defense Fund, aggregate studies conducted with Louisiana statistics demonstrated significant associations between cancer mortality and the use of chlorinated surface water (*5*). These results were significant primarily for the urinary tract and gastrointestinal cancers and were race- and sex-dependent, being primarily observed in white males. Bladder cancer in particular showed a strong association with drinking water among white males.

The present study uses the last half of the same data base as used for the aggregate studies and includes 6 later years, but is a case-control mortality study in which the residence and water supply of each decedent was individually linked. The methodology chosen allowed for greater specific control over certain known confounders such as urban or industrial influences, though many life-style characteristics were still unknown and therefore uncontrolled. So while this study provides more certainty than the aggregate studies which preceded it, it cannot provide final proof of a

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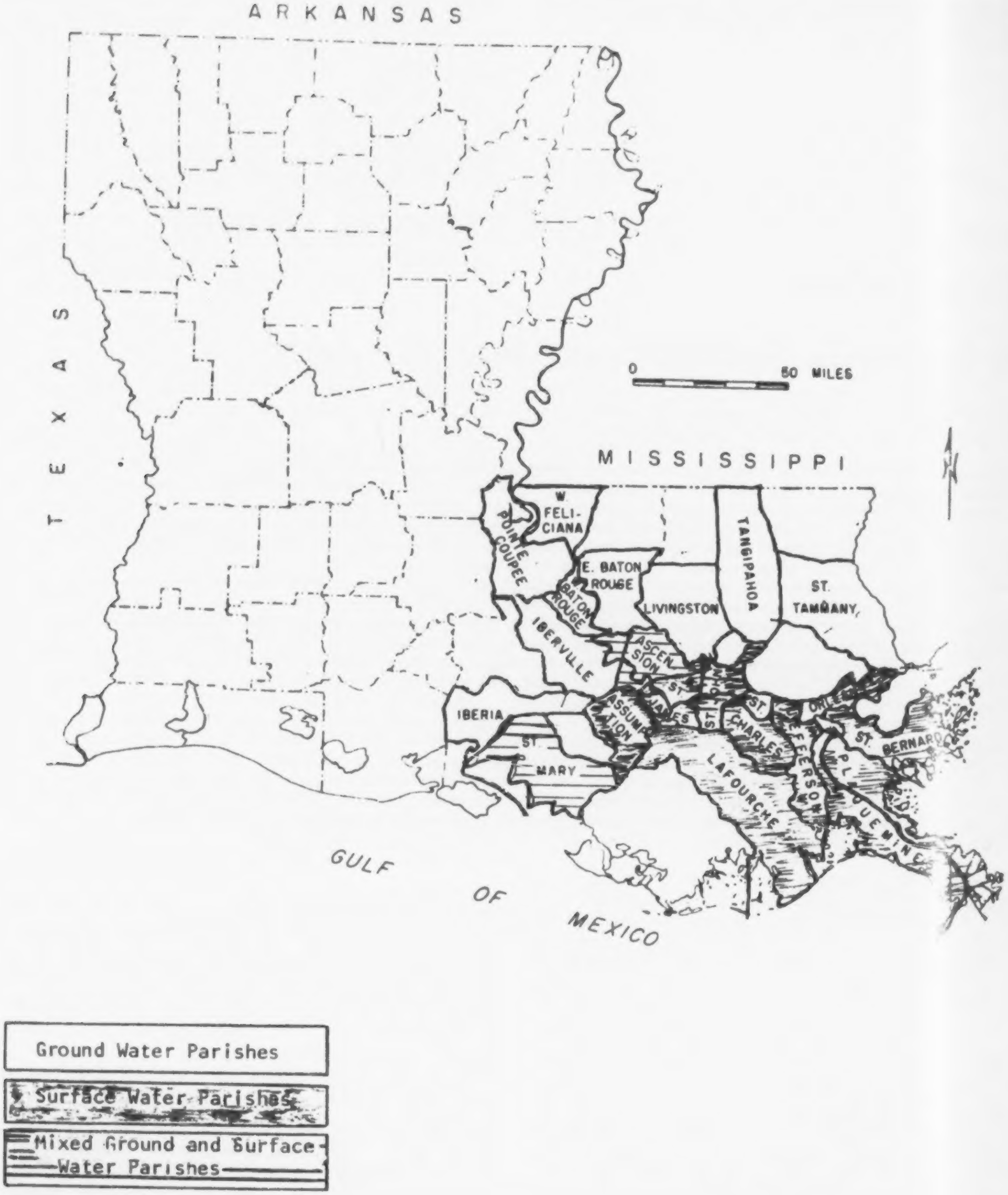
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ST. JOHN
ASSUMPTION
IBERIA
ST. MARY
ST. CHARLES
LAFOURCHE
JEFFERSON
ORLEANS
ST. BERNARD
PLAQUEMINES
GULF OF MEXICO
Ground Water Parishes
Surface Water Parishes
Mixed Ground and Surface Water Parishes

FIGURE 1. Louisiana parishes included in the study and their water source.

drinking water effect on the mortality from any given cancer.

Methods

Figure 1, a map of Louisiana, illustrates the parishes (counties) included in the study and their water source.

As shown in Table 1, 13 parishes (counties) in South Louisiana were combined into groups which were similar in industrial characteristics but contained parishes using ground and surface water in approximately equal proportions. The parish groupings and their 1970 populations are presented in Table 1. Cases were cancer deaths from each of 17 cancer sites, in some of which a drinking water risk was expected and sites where none was expected. Sample size was determined by the magnitude of the expected risk based on the previous studies using aggregate data or by the available population, whichever was smaller. The sample size used for each cancer site is presented in Table 2, with the ICDA numbers which defined the sites used. For the first eight cancer sites, the population used represents all six parish groups, while for the last nine cancer sites only three of the six parish groups were used. The deleted three parish groups together represented only about 20% of the total study population and were methodologically problematic for various reasons. Therefore, they were not included for the second half of the cancer sites.

An equal number of noncancer deaths of the same race, sex, year of death and age as the case were randomly selected from within the noncancer deaths in the parish group in which the case was a resident. Death certificates for all of the cases and controls were abstracted for information on usual occupation and industry and location of residence. Location of the residence at death was used to identify the water company or water source in use at that residence. Water companies supplied service area maps and also indicated which residences utilized private wells. Length of residence at the residence at death was unknown and attempts to determine it through external sources were not uniformly successful.

Table 2 presents the number of cancer deaths in the 13 study parishes between 1960 and 1975 for each of the 17 cancer sites studied. The total number of cancer deaths selected, after sampling Orleans by 1/3, was 19,936. From these, 11,349 were used for the study, which when matched with controls resulted in a total population of 22,698.

The drinking water variables used were acquired from the Louisiana State Office of Health Services and Environmental Quality and reflected the levels available for the water source in use at death and for the years immediately preceding the year of death, going back in time as far as the data permitted. In examining chlorine, the population was trichotomized into those whose water source was nonchlorinated (groundwater), those whose

Table 1. Population resident in study parishes by parish group.

Parish group	Water source	Parish	1970 Pop.	Total
A	Ground	St. Tammany	63,685	
	Surface	Lafourche	68,941	132,626
B	Ground	Iberville	30,746	
		Iberia	57,397	
		Livingston	36,511	
	Surface	St. Charles	29,550	
		St. Bernard	51,185	
		Plaquemines	25,225	
		St. James	19,733	250,347
C	Ground	East Baton Rouge	285,167	
	Surface	Jefferson	337,568	622,735
D	Ground	West Feliciana	11,376	
	Surface	Assumption	19,654	31,030
E	Ground	West Baton Rouge	16,864	
		Tangipahoa	65,875	
		Pointe Coupee	22,002	
	Surface	Orleans (1/3)	197,823	302,564
F	Mixed	Ascension	37,086	
		St. John	23,813	
		St. Mary	60,752	132,626
Total				1,460,953

Table 2. Cancer sites included in the study, total available deaths and sample size.

Cancer site	I.C.D.A. codes 7th. revision	 8th. revision	Total no. deaths	Sample size
Liver	155.0, 156.1-156.2	155.0-155.1, 197.8, 197.7	696	548[a]
Brain	193.0, 193.9	191.0-192.9	650	611
Pancreas	157	157.0-157.9	1498	980
Bladder	181.0, 181.8	188	759	759
Kidney	180	189.0-189.2	482	440[b]
Prostate	177	185	1400	932
Rectum	154	154.0-154.2	724	692
Colon	153.0-153.9	153.0-153.9	2402	1167
Esophagus	150	150	457	457
Stomach	151	151.0-151.9	1391	700
Non-Hodgkins' lymphoma	200.0-200.2, 202.0-202.1, 205	200.0-200.1 202.0-202.9	538	531
Multiple myeloma	202	203	273	267
Leukemia	204.0-204.4	204.0-207.9	1033	989
Hodgkin's lymphoma	201	201	248	236
Lung and bronchus	162.1, 163	162.1	5221	880
Breast	170	174	1970	968[c]
Malignant melanoma	190.0-190.9	172.0-172.9	194	192
Total			19,936	11,349

[a] Excluding definitely secondary liver.
[b] Excluding Wilms' tumor cases.
[c] Excluding males, in addition to sampling.

water source had a chlorination level below the mean (which was 1.09 ppm) and those whose water source had a chlorination level above the mean. The chlorination level used was measured from the finished water as it left the water treatment plant, rather than as it came out of the tap.

Since it was possible to assign the chlorine variable to only about 80% of the study population, a possibly biased lost-to-follow-up must be considered. In addition, the value was highly associated with year of death, so it is likely that the recording or the use of chlorine changed during the study period. The use of the value recorded at the treatment plant does not capture the loss of chlorine concentration as it travels different distances throughout the supply system. The other way of looking at chlorine level in a more qualitative manner, by separating water source into ground nonchlorinated, ground chlorinated and surface chlorinated, has the advantage of using the total study population and possibly reflecting interactions between contaminants of the surface water source and chlorination. This qualitative distinction will be used to examine any findings which suggest a purely chlorine or chlorine in conjunction with a particular source of water.

In order to evaluate the effect of chlorination on the distribution of cases and controls in the light of other variables which might be significant effect-modifiers, a multidimensional contingency table analysis was performed which was based on the log-linear model and utilized maximum likelihood estimations of all main effects and interactions. This is a BMD package program, P3F (*6*). By using this method of analysis it is possible to see to what extent all main effects are dependent on higher order interactions. All of the matching variables were used as possible effect modifiers in the analysis, with age dichotomised at the mean for the site, and year of death, dividing the study period in halves. Chlorine was trichotomized into none, low or high and its relationship with disease, or case/control, was examined by sex, race, age, and year of death. By using the methodology recommended by Morton Brown, the basic structure of the data was explored by fitting different explanatory models and determining the model with the closest fit (*6*). The first model fit was all of the highest order interactions, and from this all interactions were deleted which did not make a significant contribution to the overall fit. By this process a final model was developed composed of various levels of interactions, which as a whole described the data optimally given the variables. The contribution of each of the terms of the model to the overall fit is estimated by a likelihood ratio chi square.

Results

Table 3 presents the results from the multi-way contingency table analysis for each of the different cancer sites when analyzed with chlorine level. The

Table 3. Summary of multiway contingency table analysis for cancer sites by chlorination of water.

	Chlorination level (none, low or high)		
	Significance level of		
Cancer site	Main effect	Higher order	Significant effect modifiers
Liver	—	—	—
Brain	—	0.008	Race, age, year of death
Pancreas	—	0.047	Race, age, year of death
Bladder	—	—	—
Kidney	—	0.010	Sex, age, year of death
Prostate	—	.045	Race, age, year of death
Rectum	0.012	—	None
Colon	—	—	—
Esophagus	—	—	—
Stomach	—	0.018	Sex, race, age
Non-Hodgkin's lymphoma	—	—	—
Lung	—	—	—
Breast	—	—	None

significance level of the main order effect, of chlorine and disease unmodified by any of the other variables, is presented in the first column. Rectum and breast are the only cancer sites which show a significant main effect. The next column shows the significance level of higher order interactions, in which the correlation of disease and chlorine was dependent on other variables. These other variables, effect modifiers, are listed in the last column. Brain, pancreas, kidney, prostate and stomach show five-way interactions between disease, chlorine and three other variables. Table 4 shows the results for the sites where the qualitative distinction of water source type was used rather than the chlorine level. This was done because of the small sample size of many of these sites. Only malignant melanoma shows a significant higher order interaction in which disease and water source is dependent on sex and age.

Brain cancer showed a highly ($p = 0.008$) significant relationship between disease, chlorine level, race, age and year of death. On examining the distribution it was discovered that there was a chlorine effect but it was seen only among the younger whites. Table 5 shows the distribution of young whites by chlorination level for the first half of the study period and for the last half of the study period. Odds ratios are calculated by comparing each chlorination level to no chlorine. There is a chlorination effect but it is not dose-dependent, that is, the low chlorine level shows more risk than the high chlorine level. The later time period shows a higher risk than the earlier time period. In fact, when this group is analyzed by the contingency table analysis, the main effect between disease and chlorine is significant among this group, although not among the earlier group.

While it is difficult to explain why chlorination would affect whites more than blacks, the association of the risk among those younger than the mean age, which in brain is 46 years, and its appearance in the later time period is cause for concern. While a dose-response relationship would be added validation to this effect, the chlorination level is sufficiently imprecise to understand the lack of such a relationship. When the effect is examined by the qualitative variable of water source type, the risk is found among the ground chlorinated category exclusively

Table 4. Summary of multiway contingency table analysis for cancer sites by type of water source.

	Water source type (surface, ground Chl, ground non-Chl)		
	Significance level of		
Cancer site	Main effect	Higher order	Significant effect modifiers
Multiple myeloma	—	—	—
Leukemia	—	—	—
Young	—	—	—
Old	—	—	—
Hodgkin's lymphoma	—	—	—
Malignant melanoma	—	0.04	Sex, age

Table 5. Distribution of white cases and controls younger than the mean age by chlorination level and year of death and brain cancer.

Chlorination level	Year of death < 1969			Year of death ≥ 1969		
	Cases	Controls	O.R.[a]	Cases	Controls	O.R.[a]
High chlorine (> 1.09)	23	14	1.73 (0.64-4.70)	25	18	1.97 68-5.72)
Low chlorine (≤ 1.09)	33	19	1.82 (0.73-4.56)	23	7	4.65 34-16.89)
No chlorine	20	21	—	12	17	

[a] Odds ratio between each category and no chlorine.

and not among the surface chlorinated. In addition to these results with water an analysis of occupation turned up a significant risk for brain cancer in chemical workers, with a lower mean age in these cases (51 years) than in the controls (62 years) who worked for chemical plants. Since the Baton Rouge area has many chemical and petrochemical industries, and is also the largest area using ground chlorinated water in this study, it may be that this chlorine effect is actually due to confounding with occupation. This could explain the fact that the effect is seen only among whites and may represent an occupational bias.

In pancreatic cancer, the multidimensional contingency table analysis uncovered a five-way interaction between disease, chlorine, race, age and year of death. On examining these effects in detail the interaction term, which was only of borderline significance, represented an extremely inconsistent pattern. Risks for both nonchlorination and for high chlorination were seen in different cells of the table, with no consistent trend in other cells. On balance it appears that no association of pancreatic cancer and chlorination level is demonstrable from these data.

Bladder cancer showed no relationship with chlorination or with any of the other water variables. However, because of the results of the Environmental Defense Fund report in 1974, where a significant water effect was observed for white males, the data was examined more closely to see if an effect could be detected. In Parish Group B, consisting of rural parishes above and below the Mississippi River from New Orleans (Orleans, Jefferson and St. Bernard Parishes), there was a highly significant surface water risk among white males, as seen on Table 6. The number of parishes involved in this risk is large, though the population of the parishes is small. In a multiple regression procedure with each parish contributing equally, the effect of these parishes is much larger than in a methodology based on individuals and their water source. It is possible that the pattern seen in these parishes may have been overemphasized by the method of analysis chosen previously. It appears unlikely that there is a surface water contaminant in the water above and below New Orleans but not in New Orleans, which only affects white males. This result is likely to be one of the random significances one expects to see in subsets of a large data set, or confounding associated with another environmental factor.

Table 6. Distribution of bladder cancer cases and controls by parish in parish group B white males.[a]

Water source	Parish	Cases	Controls
Ground	Iberia	9	17
	Iberville	10	10
	Livingston	14	22
	Total	33	49
Surface	Plaquemines	6	2
	St. Bernard	13	7
	St. Charles	8	4
	St. James	4	2
	Total	31	15

[a] Odds ratio (surface to ground): 3.07; $p < 0.01$.

Kidney cancer also showed a highly significant five-way interaction between disease, chlorine, sex, age and year of death, but on examination of specific cells, this too was discovered to represent an inconsistent pattern. The only significant risk seen was for nonchlorination among older males dying in the earlier half of the study period.

Although a slight risk for chlorination was observed in all of the other cells of the table, this risk was not sufficiently pronounced to be significant. Prostate showed the same pattern of inconsistencies between cells of the table, with the only significant risk being for no chlorination among young whites dying in the earlier half of the study period.

With rectal cancer, however, there is a significant main effect between disease and chlorine which is not dependent on any of the other variables in the model. Table 7 shows the distribution of the cases and controls by chlorination level, with the odds ratio of each chlorination category compared with the nonchlorine category. There is a dose-response

Table 7. Distribution of rectal cancer cases and controls by water source type and chlorination level.

Chlorination level	Cases	Controls	O.R.[a] (95% C.I.)	Water source type	Cases	Controls	O.R.[a] (95% C.I.)
High (> 1.09 ppm)	176	135	1.68 (1.17-2.42)	Sur, Chl.	462	396	1.53 (1.15-2.04)
Low (≤ 1.09 ppm)	274	275	1.29 0.93-1.79)	Gnd, Chl.	106	134	1.04 (0.72-1.50)
No	96	124		Gnd, Non-Chl.	116	152	
Total	546	534		Total	684	682	

[a]Odds ratio between each category and no chlorine.

Table 8. Distribution of rectal cancer cases and controls by chlorine level for those on surface water.

	Surface water		
Chlorination level	Cases	Controls	O.R.[a] (95% C.I.)
High (> 1.09 ppm)	175	133	1.12 (0.81-1.55)
Low (≤ 1.09 ppm)	181	154	
Total	356	287	

[a]Odds ratio of high to low chlorine.

Table 9. Distribution of rectal cancer cases and controls by water source for those on low chlorine.

	Low chlorine		
Water source type	Cases	Controls	O.R.[a] (95% C.I.)
Surface	181	154	1.53
Ground	93	121	(1.07-2.19)
Total	274	275	

[a]Odds ratio of surface to ground.

relationship, with the low chlorine showing less of a risk than the high chlorine category. When this same relationship is examined using the qualitative variable of water source type, however, it is clear that the difference is found mostly between surface and ground water, since there are more controls than cases in the ground chlorinated category.

Because surface water source and heavy chlorination are so closely related it is difficult to distinguish between their effects. However, one can first control for chlorination and look at residual risk for water source, and similarly control for water source and look at residual risk for chlorination. In the surface water category there is some variability in the level of chlorination, and Table 8 shows the distribution of cases and controls by chlorine level among those on surface water. While there is a slight increased risk in the high chlorine category, this increase is not statistically significant. The surface water effect among those in the low chlorine category is presented in Table 9 and demonstrated that there is still a significant surface water effect after controlling for chlorine level, though it is less than it was when not controlling for chlorination.

Because controlling for water type diminishes the risk for chlorine and controlling for chlorine diminishes the risk for surface water, it is likely that both are necessary for the large increased risk seen for chlorinated surface water. This is, of course, an intuitively attractive hypothesis since the potential for damage from chlorination is so much greater if the water is heavily contaminated with organics before being chlorinated. While it is methodologically impossible to separate heavy chlorination from surface water source in South Louisiana, it is clear that whether it is due to the chlorination per se or to the chlorination added to contamination, the surface chlorinated water of South Louisiana is definitely associated with a significant risk for rectal cancer.

No risk whatsoever was observed with colon cancer, in contrast to rectal cancer, though control selection and data processing for these two sites were done simultaneously. The fact that colon cancer does not associate with drinking water at all though it is known to be highly correlated with lifestyle factors such as diet, supports the assumption that in fact the methodology used for this study was successful in controlling for these other factors.

Stomach cancer also showed a five-way interaction between disease, chlorine, sex, race and age. This was found to consist of a risk for high chlorine among older white males, but no such risk was detected in the other cells of the distribution and therefore was judged to be inconsistent. No chlorine effect was observed with lung or with non-Hodgkins' lymphoma.

Breast cancer, however, showed a significant main effect between chlorine level and disease. The distribution of cases and controls is presented in Table 10 along with the odds ratio between each

Table 10. Distribution of breast cancer cases and controls by chlorination level.

Chlorination level	Cases	Controls	O.R.[a] (95% C.I.)
High chlorine (> 1.09 ppm)	309	294	1.58 (1.09-2.29)
Low chlorine (≤ 1.09 ppm)	489	457	1.61 (1.13-2.30)
No chlorine	64	96	
Total	862	847	

[a] Odds ratio between each category and no chlorine.

category and the no chlorine category. As is seen from the table, the risk for low chlorine is higher than the risk for high chlorine, though both are significantly greater than one. On close examination of this risk, however, it was discovered that it was not found in the parish group consisting of Baton Rouge and Jefferson parishes, two urban parishes comprising the same parish group. That is, in the parish group where urban lifestyle was the most successfully controlled, the risk disappeared. Following that observation, the other parish groups were closely examined to see whether the observed risk might be reflecting an urban area with chlorine matched with more rural nonchlorinated areas. Through detailed examination of the actual residence of the cases and controls it was determined that such confounding might possibly have been responsible for the observed effect. Since small family size is a risk factor for breast cancer and an attribute of urban living such confounding could have resulted in an apparent risk for chlorination.

The cancer sites which were examined by using the water source type variable showed no association between disease and water, except for malignant melanoma, where there was a risk still dependent on sex and age. This consisted of a risk for ground water among older females, a risk which was not reflected in the other cells of the table.

Discussion

In conclusion, then, there is a definite association between chlorinated surface water and rectal cancer, with a risk of about 2 for those living on the chlorinated surface water. This risk is not sex- or race-dependent, or dependent on any of the other variables in the model. In results reported elsewhere (7), it was also shown that this risk increased as one approached the mouth of the Mississippi River and increased with increased duration of use. In contrast to this, colon cancer showed no association with any water variable. Rectum is a logical target site to demonstrate this effect due to its physiologic function.

The apparent main effect with breast cancer and chlorinated water may be due to confounding of small family size with chlorinated water though it might also be a valid water association. The fact that it is not present in the parish group made up of the two urban parishes raises questions about the observed effect. The association of brain cancer with chlorine is also questionable, since it is present only among whites and primarily in the Baton Rouge area, where there are many chemical plants. Association between chlorination and other sites of cancer are heavily dependent on the matching variables of race, sex, age and year of death and the effects observed were not found to be consistent in any way which would suggest a true drinking water effect.

While those apparent effects appear to be nonsubstantial, the results with bladder show a clear lack of drinking water effect though there is a risk for drinking surface water in one parish group in one race/sex category. Since the risk seen is from parishes above and below New Orleans, but not in the New Orleans area itself, it is unlikely to be a drinking water risk, but more likely an industrial risk.

Therefore, there appears to be some risk associated with water chlorination, although some definitive and specific studies must be undertaken especially with regard to the importance of co-contaminants and possible industrial confounders. Recently reported similar observations by Kanarek and Young with regard to colo-rectal cancer and chlorinated drinking water are supportive of these observations (*8*). Although the risk for colon cancer was not observed in this study, this could be attributed to methodologic differences.

The fact that only rectal cancer demonstrates this association even on such a survey study as this, is likely an indication of risk to other organ sites which a more definitive study is necessary to demonstrate.

This paper was presented in part at the Society for Epidemiologic Research Meeting, June 1981.

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Environmental Health Perspectives
Vol. 46, pp. 179-186, 1982

Drinking Water Treatment and Risk of Cancer Death in Wisconsin

by Marty S. Kanarek* and Theresa B. Young†

A case control study of drinking water treatment practices and female cancer mortality was conducted in Wisconsin. Cancer deaths for 1972-1977 from 28 Wisconsin counties and noncancer deaths matched to cancer deaths on age, year of death and county of residence, were compared for characteristics of drinking water supplied to their places of residence. Using logistic regression, estimates of relative risk associated with chlorinated water were examined allowing for the influence of indicators of water organics and the potential confounders of occupation, marital status and urbanicity. Only colon cancer appeared to be related significantly to chlorination in all models explored. A dose-response relationship was found between crude indicators of trihalomethane level (chlorination × organic contamination) and colon cancer death. The odds ratio for chlorinated surface water for colon cancer was 2.81 ($p < 0.01$); approximately half this risk was found for chlorinated ground water. Consequently, a case control study of colon cancer and drinking water quality utilizing newly diagnosed patients is being conducted in Wisconsin.

Introduction

In recent years, the human cancer risk associated with chronic, low-dose exposure to trihalomethanes (THMs) present in drinking water has been the ultimate concern of diverse research. [Trihalomethanes have the structure HCR_3, where R represents chlorine, bromine or iodine, or combinations thereof; the major THMs in drinking water are chloroform, bromodichloromethane, dibromochloromethane and tribromomethane (bromoform).] After the National Organics Reconnaissance Survey (NORS) documented the pervasiveness of THMs (particularly chloroform) in U.S. municipal water disinfected with chlorine (*1*), researchers in the field of water chemistry have sought to identify THM precursors to quantify reaction conditions which explain the variance in THM concentrations found in the finished water of different facilities. On the biological front, long-term rodent studies continue to show oncogenic effects following chronic chloroform ingestion (*2*). Preliminary epidemiologic studies of water quality and cancer mortality have associated some gastrointestinal and urinary tract cancers with presumed exposure to water likely to contain THMs.

As discussed in recent reviews (*3,4*), the epidemiologic studies vary in the specificity of exposure classification with respect to THMs. Since THM levels are unknown for the exposure period relevant to current cancer deaths (e.g., 15–20 years prior to disease onset to allow for latency), all exposure classification schemes are necessarily indirect and consequently rest on a set of assumptions. In the final analysis, it is the explicit or implicit model which holds the key to the assumptions inherent in a particular study, the biases introduced, the limitations and specificity, and the nature of the conclusions which may be drawn. This is a report of the epidemiologic model, methods and further results of the Wisconsin case control study of female gastrointestinal, urinary tract, brain, lung and breast cancer mortality and drinking water trihalomethanes. Previous analysis indicated that only colon cancer death was significantly associated with exposure to chlorine-dosed water (*5*). The odds ratio estimate of approximately 1.50

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did not appear to vary by chlorine dose level but was significantly modified by stratifying the sample on probable presence or absence of halogenatable organics in the water source. For the subsample with raw water affected by rural runoff, risks of 2.74, 3.60 and 3.30 for low, medium and high chlorine dose levels, respectively, were found. Since crude organic precursor indicators seemed to have a greater effect on colon cancer risk than dose level, an analysis based on crude organic levels of chlorinated water was conducted and is reported here.

Methods

Epidemiologic Model

For the investigation of Wisconsin female cancer mortality and water exposure, an attempt was made to make use of all historical water data on parameters shown by prior research to be relevant to THM formation. These water characteristic and treatment parameters, other factors related to THM exposure, and potential confounders with respect to water source and cancer mortality are included in the epidemiologic model presented in Figure 1. This model has guided the investigation and serves as a backdrop for the description of methods and analysis employed, and discussion of results.

Section I of the model pertains to raw water concentration of halogenatable organics, or THM precursors. Naturally occurring organics can be considered as a function of water source, with surface source and shallower wells likely to contain higher concentrations. Water color and turbidity are indirect indicators of "humus." Rural runoff which impinges on water source is considered the most important source of natural organics, while industrial discharge, municipal waste and air pollution are possible other sources of halogenated or halogenatable organics. It is important, too, to consider these conditions as possible sources of toxicants which potentiate or are potentiated by THMs.

Section II represents water treatment and reaction conditions known to influence THM formation. Chlorination dose has been shown to be an important predictor of THM concentration; both prechlorination, and post or one-step chlorination are considered here in addition to the total dose for the two procedures combined. Other factors include pH, water temperature, and purification procedures (coagulation, sedimentation, and filtration).

Actual THM exposure is represented in Section III, where amount of daily water consumption and chronicity of exposure to a particular source is considered. Information on THM exposure from sources other than drinking water is extremely limited. Calculations of "typical" uptake of chloroform (in mg/year) from water, food, and ambient air indicate that uptake from water is 2–3 times greater than that from the other sources (*6*). However, when minimum uptake is considered (lowest range for "reference man"), uptake from the atmosphere is ten times greater than that from water. One potential source which may be of importance for young age groups is that of chlorinated swimming pools, where THM levels of up to 1400 μg/l. have recently been detected (*7*).

At the next point in the model, Section IV, the

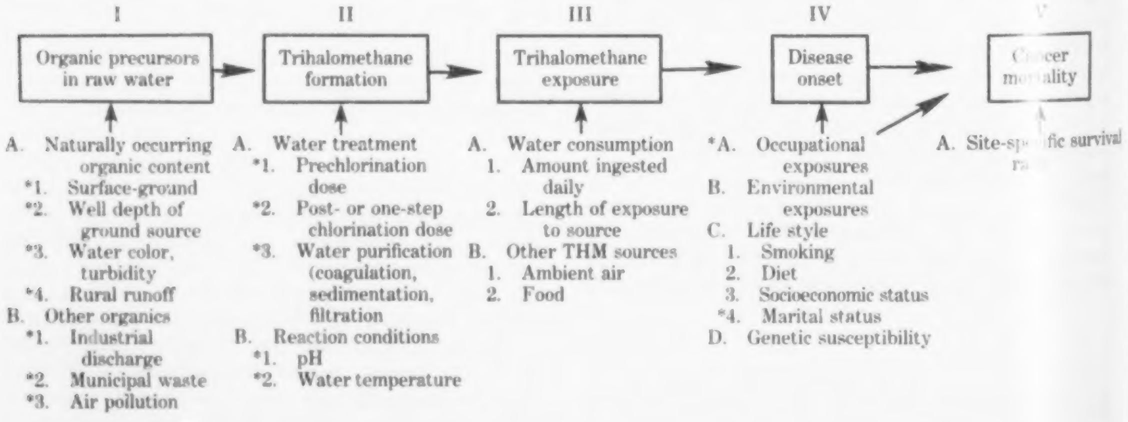
I
II
III
IV
V
Organic precursors in raw water
Trihalomethane formation
Trihalomethane exposure
Disease onset
Cancer mortality
A. Naturally occurring organic content
*1. Surface-ground
*2. Well depth of ground source
*3. Water color, turbidity
*4. Rural runoff
B. Other organics
*1. Industrial discharge
*2. Municipal waste
*3. Air pollution
A. Water treatment
*1. Prechlorination dose
*2. Post- or one-step chlorination dose
*3. Water purification (coagulation, sedimentation, filtration
B. Reaction conditions
*1. pH
*2. Water temperature
A. Water consumption
1. Amount ingested daily
2. Length of exposure to source
B. Other THM sources
1. Ambient air
2. Food
*A. Occupational exposures
B. Environmental exposures
C. Life style
1. Smoking
2. Diet
3. Socioeconomic status
*4. Marital status
D. Genetic susceptibility
A. Site-specific survival rate

FIGURE 1. Epidemiologic model of trihalomethane exposure and cancer mortality. Data on the factors marked with an asterisk (*) were collected for this investigation.

influence of coexposures and confounders must be considered. Many factors, such as hazardous industrial exposures, may be related to both cancer incidence and living in areas served by chlorinated water. It is also possible that some of these factors act synergistically with THMs.

Since this study, as most reported to date, takes cancer death as the measured effect, it is important to consider causal and confounding factors related to cancer death. While influences on this section of the model (V) are largely unknown and unquantifiable, the assumptions and limitations so introduced by use of mortality data must be addressed.

Study Design

A case control study utilizing mortality data was conducted. The characteristics of water supplied to the usual place of residence (as listed on the death certificate) of cancer decedents were compared to that of noncancer decedents. In order to maximize the necessary assumption that the cases and controls were exposed to the water supply associated with the "usual place of residence" listed on the death certificate for 15–20 years before death, only counties with less than 10% population increases attributed to immigration over the past 20 years were included in the study. In addition, the study was restricted to females, and since females of this cohort of decedents (death years 1972–1977) were likely to have been homemakers, more exposure to the home water supply is likely.

The sample of white female cases and controls was obtained from computer tapes of abstracted death certificate data, provided by the Wisconsin Department of Health and Social Services, Bureau of Health Statistics. Only deaths from 28 of the 72 Wisconsin counties were considered. In addition to the criteria of low immigration rates mentioned above, counties had to have both chlorinated and unchlorinated municipal water supplies. Due to the lack of racial heterogeneity in most of Wisconsin, only white female deaths were included. Based on Wisconsin female cancer death rates, mortality data for six years were determined from sample-size tables (*8*) to be adequate for detecting a site-specific relative risk of 1.5 (at an error level of $\alpha = 0.05$, $\beta = 0.1$); 1972–1977 was the most current six-year period for which abstracted death certificate data were available.

Cases were defined as all white female deaths occurring 1972–1977 within the 28 study counties due to malignant neoplasms of the following sites: esophagus, stomach, colon, rectum, liver and intrahepatic bile ducts, pancreas, urinary bladder, kidney, lung, brain and breast. Each case was matched with a female noncancer death control on year of birth, county of residence and nearest birth date (day, month, year). After matching on death year and county, 95% of the cases were matched within the same year of birth as the control, 4.5% were matched within a three-year interval, and 0.5% within a six-year interval. For the majority of controls (70.93%), the cause of death was in the category of circulatory system disorders.

Several water variables were constructed for use in a statistical model, and the necessary data from all waterworks ($N = 202$) serving the 28 study counties were gathered. Individual exposure data were then assembled by assigning each case and control the values of the water variables for the particular waterworks which served the place of residence (city, town, or village) of the case or control recorded on the death certificate.

For each waterworks, data on type of water source (surface/ground), depth of ground source in feet, and use of purification procedures (coagulation, sedimentation, and filtration) were obtained from the 1970 Wisconsin Waterworks Survey report (based on data for the period 1960–1965). More detailed information on chlorination doseages used over the past 20 years (average daily dose in ppm) and the occurrence of several environmental factors influencing organic content of raw water (rural runoff, industrial discharge, air pollution, water with taste or color) were ascertained from a mail-back questionnaire sent personally to each water superintendent.

Data on the potential confounding factors of urban residence, occupation and marital status were based on death certificate information and assigned to each case and control. For urbanicity, community of residence was ranked into one of six population size categories on the basis of the 1960 census. Cases or controls with occupations considered high risk for each particular cancer site were given a score of one, otherwise zero. The marital status variable indicated married or not married.

To estimate the site-specific cancer risk associated with water supply, several statistical models were analyzed, using logistic regression. The distinct advantage of this technique lies in the ability to take factors into account which may confound the association of interest as well as allowing for the influence of factors related to the strength of the association (*9*). Thus, when variables for potential confounders are included in the logistic regression model, the estimated value of the regression coefficient for site-specific cancer deaths in relation to chlorination (a direct estimate of the odds ratio associated with the exposure) is considered to be adjusted for the other variables in the model (*10*).

The basic model (Model I) used to test the null hypothesis that cancer death is not associated with chlorinated drinking water is specified in Eq. (1) with urbanicity, marital status and site-specific high risk occupation included as potentially confounding variables.

Risk of cancer death with chlorination, controlling for confounding variables:

$$\mathrm{Log}\left[\frac{p\ (\text{chlorination})}{1-p\ (\text{chlorination})}\right] = \alpha + \beta D + \delta \mathbf{Z} \qquad (1)$$

where

D = Cancer death (yes = 1, no = 0)
Z_1 = Urbanicity (6 population categories)
Z_2 = Marital status (unmarried = 0, married = 1)
Z_3 = Cancer site-specific high risk occupation (yes = 1, no = 0)
Z_4 = Age (in years)
Z_5 = County chlorine exposure (%)

Since the variance in THM content of chlorinated water is expected to be a function of the presence of halogenatable organics, a more specific model [Model II, Eq. (2)] was tested which included terms for the presence of organic contamination, water purification and water source depth (surface or shallow well = 0, deep well > 150 ft = 1). The odds ratios for risk of cancer death and chlorination which result from this model are thus considered adjusted for confounding and exploratory variables.

Risk of cancer death with chlorination, controlling for confounding and explanatory variables:

$$\mathrm{Log}\left[\frac{p\ (\text{chlorination})}{1-p\ (\text{chlorination})}\right] = \alpha + \beta D + \delta \mathbf{Z} + \gamma \mathbf{W} \ (2)$$

where

D, $\mathbf{Z}$ are as for Eq. (1),
W_1 = Organic contamination from any source (yes = 1, no = 0)
W_2 = Water purification (yes = 1, no = 0)
W_3 = Source depth (surface or wells < 150 ft = 0, > 150 ft = 1)

A third set of models [Model III, Eq. (3)] were used, which feature categorical chlorination-organic exposure variables. Under these models, risk estimates are calculated for categories expected to more closely approximate THM content.

Risk of cancer death with organic-chlorination exposure categories, controlling for confounding and explanatory variables:

$$\mathrm{Log}\left[\frac{p\ (\times\ \text{exposure})}{1-p\ (\times\ \text{exposure})}\right] = \alpha + \beta D + \delta \mathbf{Z} + \gamma \mathbf{W} \ (3)$$

where

D, $\mathbf{W}$, $\mathbf{Z}$ are as above, and with the exposure categories given in Table 1.

Results

Water treatment for the 28 study counties is summarized in Table 2. Relevant correlations between water variables for raw water characteristics and treatment are given in Table 3. As expected, high chlorine dose is strongly and positively associated with point and nonpoint sources of organic contamination, surface source, and large population of community; and negatively associated with depth of ground source. Waterworks serving greater populations, or drawing surface or shallow water are more likely to report more sources of organic contamination. It is noteworthy, however, that chlorine dose and presence of organic contamination are also positively associated with water purification. Since purification may be expected to reduce THM content, it is important to consider these interrelationships.

The odds ratio estimates under model I and model II are given in Tables 4 and 5, respectively. Only colon cancer was significantly related to chlorinated water exposure under model I. When the water factor terms were included (model II), the odds ratio for brain cancer increased considerably and reached significance. These two sites were then examined for consistency and with regard to the scheme of THM formation using the more complex exposure variables under model III. The resulting odds ratio estimates are presented in Table 6.

For colon cancer, the greatest risk is seen with exposure to chlorinated surface water. In addition, chlorinated water with organic contamination carries a greater risk than chlorinated water with no organic contamination. The risk of purified chlorinated water and unpurified chlorinated water differed little. It is likely that the correlation between water purification and surface water may be responsible. Alternatively, various purification procedures, grouped together in this study, may differ in their ability to effect a change in final THM content. The

Table 1. Model specifications.

	Model	Exposure categories
A.	Point and non-point organic contamination × chlorination	Chlorinated water, organic contamination Chlorinated water, no organic contamination Unchlorinated water
B.	Water source × chlorination	Chlorinated water, surface source Chlorinated water, shallow well (≤ 150 ft) Chlorinated water, deep well (> 150 ft) Unchlorinated water
C.	Water purification × chlorination	Chlorinated, unpurified water Chlorinated, purified water Unchlorinated water

Table 2. Characteristics of water supplies: 202 waterworks.

Water variable	Percent of waterworks
Chlorine disinfection	47%
Filtration, purification, sedimentation	32%
Prechlorination	20%
Surface source	20%
Rural runoff	18%
Industrial discharge	12%
Air pollution	6%
Taste or odor problem	19%
Wind	12%
Sewage	7%
Average daily prechlorination dose, ppm	0-4.80
Average daily chlorination dose, ppm	0-6.00
Well depth, ft	40-1800

results for brain cancer are most striking when the chlorination × organic contamination exposure variable was used. The estimates with source depth × chlorination as exposure are neither consistent nor significant and the estimates based on the third exposure variable, purification × chlorination, are barely significant.

Table 3. Correlations among water treatment and raw water characteristics of 202 waterworks serving the study sample of cases and controls.

	r^a
Total average daily chlorination dose with:	
Purification	0.508
Surface source	0.521
Rural runoff	0.528
Industrial discharge	0.340
Air pollution	0.273
Taste, odor problems	0.513
Wind	0.311
Sewage	0.226
Total no. of sources of organic contamination	0.452
Urbanicity	0.459
Well depth of ground source	-0.261
Total no. of sources of organic contamination:	
Purification	0.557
Prechlorination	0.797
Surface source	0.770
Well depth	-0.301
Urbanicity	0.512
Surface source:	
Rural runoff	0.732
Industrial discharge	0.644
Air pollution	0.418
Taste and odor problems	0.744
Wind	0.418

[a] All $p < 0.01$

Table 4. Odds ratios for site-specific cancer death and chlorination (controlling for confounding variables, model I estimates).

Site	Chlorinated	Unchlorinated	Total	OR	Significance (p)
Esophagus	161	33	194	0.77	0.8
Stomach	699	211	910	1.45	0.2
Colon	2565	619	3184	1.43	<0.02
Rectum	601	177	778	1.47	0.2
Liver	569	169	738	1.13	0.7
Pancreas	1069	325	1394	1.21	0.4
Kidney	368	116	484	0.52	0.2
Bladder	358	100	458	1.24	0.6
Lung	569	169	738	1.13	0.7
Brain	312	116	428	2.29	0.1
Breast[a]	1424	391	1815	0.74	0.2

[a] 3 years of data (1976-1978).

Table 5. Odds ratios for site specific cancer death and chlorination (controlling for confounding variables and organic contamination, source depth, and water purification; model II estimates).

Site	Chlorinated	Unchlorinated	Total	OR	Significance (*p*)
Esophagus	161	33	194	0.30	0.3
Stomach	699	211	910	1.30	0.4
Colon	2565	619	3184	1.46	0.02
Rectum	601	177	778	1.59	0.2
Liver	569	169	738	1.29	0.5
Pancreas	1069	325	1394	1.52	0.2
Kidney	368	116	484	0.48	0.2
Bladder	358	100	458	1.40	0.5
Lung	569	169	738	0.87	0.6
Brain	312	116	428	4.71	0.03
Breast	1424	391	1815	0.77	0.3

Table 6. Odds ratio (OR) estimates of cancer death by chlorination-organics categories (controlling for confounding and additional water variables; model III estimates).

Cancer	Category	*N*	OR	*p*
Colon	Exposure variable = organic contamination × chlorination			
	Chlorinated, organic contamination	1974	1.81	0.03
	Chlorinated, no organic contamination	253	1.41	0.03
	Unchlorinated	463		
	Total	2690		
	Exposure variable = source depth × chlorination			
	Chlorinated surface	1983	2.81	0.01
	Chlorinated shallow (≤ 150ft)	72	1.34	0.2
	Chlorinated deep (> 150 ft)	68	1.60	0.08
	Unchlorinated	367		
	Total	2490		
	Exposure variable = water purification × chlorination			
	Chlorinated, unpurified	350	1.52	0.05
	Chlorinated, purified	2021	1.75	0.01
	Unchlorinated	555		
	Total	2926		
Brain	Exposure variable = organic contamination × chlorination			
	Chlorinated, organic contamination	258	6.86	0.03
	Chlorinated, no organic contamination	27	2.53	0.2
	Unchlorinated	81		
	Total	366		
	Exposure variable = source depth × chlorination			
	Chlorinated surface	266	1.14	0.8
	Chlorinated shallow (≤ 150 ft)	10	1.06	0.9
	Chlorinated deep (> 150 ft)	8	1.60	0.6
	Unchlorinated	74		
	Total	358		
	Exposure variable = water purification × chlorination			
	Chlorinated, unpurified	33	4.41	0.04
	Chlorinated, purified	266	1.69	0.4
	Unchlorinated	97		
	Total	396		

Discussion

No site other than brain or colon cancer showed any significant association with chlorinated water, although elevated risks were generally seen for gastrointestinal and bladder cancer sites. While it appears that there are adequate numbers of cases and controls for all sites except esophagus, certain limitations of the death certificate approach may necessitate even larger samples. Misclassification error has been shown to reduce power (*11*); error of this type was introduced in this study when the assumption that the water supplied to the usual residence was consumed over a 15–20 year period prior to death was not met (whether due to migration or simply not drinking tap water). With regard to the findings of other epidemiologic studies implicating bladder or rectum cancer, it is important to stress that this Wisconsin study is based on white female deaths only. While confounding due to smoking, alcohol, occupation and probable migration is of less concern for this homogeneous sample relative to a sample of males, the generalizability of results is restricted.

The lack of a consistent brain cancer risk under the various models indicates that the brain cancer-chlorination association is not substantive. The strong influence of the organic contamination variable (an indirect indicator for any point or nonpoint source of organics) and the lack of influence of surface water source suggest that ground water contamination with specific organic contaminants may be responsible for the pattern of risk found. Alternatively, the inconsistent relationship may be due to an unknown confounding factor that is positively associated with some of the ground water supplies in particular areas.

The evidence from this analysis for a colon cancer risk is stronger, but methodological limitations stemming from the use of death certificate and other indirect data preclude drawing causal interpretations. It is not likely that known confounding factors or bias introduced via nonrandom misclassification error can completely account for the estimated risks since such factors (e.g., vegetable consumption) would have to persist through the more complex categorization by organic levels of chlorinated water. Since no risk was found for breast cancer, which is known to be linked positively to higher socioeconomic status, it is not likely that this potential confounding factor influenced only the colon cancer relationship. Moreover, urbanicity was always used as a control variable, thus lessening concern with this important confounding factor. The result, then, may be cautiously viewed as suggestive of a colon cancer risk with exposure to water likely to contain chlorinated organics.

In view of the results of this study and similar findings recently reported (*12*), drinking water exposure warrants consideration as a possible cause of colon cancer. We are currently conducting an epidemiologic study in which the lifetime THM water exposure of Wisconsin colon cancer patients are compared to that of a group of controls, in order to test the hypothesis that exposure to THMs in drinking water carries significant risk of colon cancer, and to determine the magnitude of risk. The subordinate goals include the following:

1. Development of a valid measure to assess lifetime THM exposure. This involves the measurement of THMs in approximately 100 Wisconsin supplies from different waterworks and the characterization of these water supplies on parameters for which historic data exist (e.g., chlorine dose, prechlorination or not, surface or ground source, depth of ground source, pH, water purification procedures). Using statistical modelling procedures, these data will be used to determine how much of the final THM concentration can be explained, or predicted, by the parameters. An index of the significant factors, weighted by the magnitude of association with THM concentration, will result, whereby a THM level will be calculated for all Wisconsin water supplies for any past period for which data on the index factors exist. Then, in conjunction with the history of all water sources to which an individual was exposed, length of exposure in years and amount of tap water consumed, estimates of individual lifetime THM exposure will be derived.

2. Exploration of the relationship between colon cancer and the interaction of THM exposure with other exposures and personal characteristics. Recent experimental evidence suggests that a variety of compounds are capable of potentiating the biological effects of chloroform. The risk associated with THM exposure, for example, may be compounded among those exposed to alcohol or pesticides. The relationship may also differ by personal characteristics such as ethnic heritage, diet considerations, and age at exposure to chlorinated water.

3. Exploration of the relationship between colon cancer and other environmental contaminants. One of the main features of a case control study in which subjects are available for interview, is the ability to assemble complete residential histories (address × length of time). Consequently, the past proximity to potential environmental hazards such as chemical dumps, heavy pesticide/herbicide land saturation, industrial discharge and air pollution will be examined as risk factors for colon cancer.

It is hoped that this case control study will further clarify the role of chlorinated drinking water in human colon cancer incidence.

This work was supported by U.S. EPA Contract C2769 NAEX, a grant from the University of Wisconsin Graduate School, and a grant from the Wisconsin Clinical Cancer Center, Cancer Control program.

We would like to thank Dr. Ray Nashold, the Director of Health Statistics for the Wisconsin Department of Health and Social Services, for providing us with the mortality data used in this study.

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Environmental Health Perspectives
Vol. 46, pp. 187-195, 1982

Epidemiological Evidence of Carcinogenicity of Chlorinated Organics in Drinking Water

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Concern has recently been voiced over possible chronic toxicity associated with chlorination of public drinking water supplies in the United States. This paper reviews the available evidence and the studies underway to further evaluate hypothesized associations between cancer risk and byproducts of chlorination. Preliminary data from measures of halogenated volatiles and personal exposure histories from respondents in a large epidemiologic study of bladder cancer are presented. These data support the use in epidemiologic studies of categorical measures of exposure and suggest that results from completed case-control studies, based on death certificates, may have underestimated the true risk of exposure to chlorination by-products. The current generation of studies which use a case-control interview design offer many advantages over earlier efforts to evaluate this issue.

Introduction

Chlorination of public drinking water supplies in the United States was first practiced in 1908 at the Boonton, New Jersey waterworks supplying Jersey City (1,2). Chlorination was initiated in response to a legal challenge that the water utility had not been meeting its contracted obligation to supply water pure and wholesome to drink. In reviewing the facts in May, 1910, after chlorination had been in place for more than a year, the judge noted that chlorination was quite effective in destroying "germs" and thereby improving the quality of the drinking water. He added:

> Upon the proofs before me, I also find that the solution described leaves no deleterious substances in the water. . . . I do therefore find and report that this device is capable of rendering the water delivered to Jersey City pure and wholesome, for the purposes for which it is intended and is effective in removing from the water those dangerous germs which were deemed by the decree to possibly exist therein at certain times (*1*).

In light of its obvious benefits, concern about adverse effects of chlorination rapidly diminished, and the practice spread to most large population centers of the United States within a decade. Introduction of chlorine disinfection came after two decades that had witnessed introduction of filtration, sedimentation, and coagulation to many large water supplies. These practices were themselves very effective in controlling levels of water-borne infectious bacteria.

Figure 1 shows the dramatic decrease in U.S. typhoid mortality between 1900 and 1928 (*3*). Introduction of filtration in public water supplies had already accounted for a large part of the decrease in mortality rates before the widespread use of chlorination in the years after 1910. In Cincinnati, for example, the annual typhoid rate dropped from 379 per 100,000 in 1905–1907 to 60 per 100,000 in 1908–1910 with the introduction coagulation, sedimentation and filtration of the public drinking water. The addition of a chlorination step a few years later further decreased the rate. Current studies are now attempting to quantify the health and economic benefits of chlorination.

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Concern over adverse effects of chlorination was quiescent from 1910 until a few years ago. In 1974, it was reported that chloroform and other trihalomethanes could be produced from the interaction of chlorine and organic molecules such as humic and fulvic acids in the raw water of drinking water supplies (*4*,*5*). A subsequent nationwide survey by the Environmental Protection Agency showed indeed that chloroform is found in almost every chlorinated supply (*6*). Concern was elevated when a National Cancer Institute animal feeding study indicated that chloroform is a carcinogen (*7*). More recently, attention has been focused on the genotoxic potential of higher molecular weight nonvolatile chlorinated organics that are also produced during chlorination (*8*).

The implications of an association of chlorination with one or more types of human cancer are immense. A recent survey from the EPA Office of Water Supply estimates that 192.2 million persons in the United States are served by community water systems (*9*). The vast majority of these systems use chlorine as a disinfectant. The health benefits to be gained by limiting, curtailing, or substituting for chlorine must be carefully balanced by considering other health risks which may result.

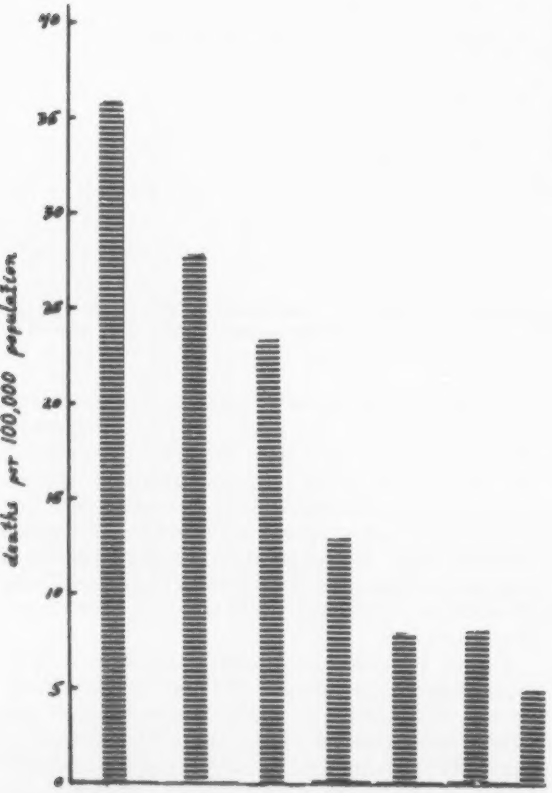

FIGURE 1. Annual U.S. typhoid mortality rates, 1900–1928.

Measures of Exposure in Epidemiologic Studies

A lack of specific historical environmental data, especially chemical measurements, has compelled the use in epidemiologic studies of surrogate measures of past exposure to water-borne organic chemicals. Several types of water quality indicators have been used. The proportion of a county's population using surface as opposed to ground water is one, and the county population served by chlorinated sources, in contrast to nonchlorinated, is another. The percentage of a county's population served by water from a river with upstream industry may be considered a special case of the surface/ground indicator. Other water quality variables used in epidemiologic studies are trihalomethane concentration in the supply serving an individual or most of a county and the historical chlorine dose in such supplies.

An environmental data base under development in conjunction with a large-scale bladder cancer case-control study provides some insight into the categorical exposure measures of epidemiologic studies. Collection of this data was supported by the Cincinnati Health Effects Research Laboratory of the Environmental Protection Agency. The first completed data come from the state of Iowa, one of ten areas of the United States in this study. We collected information and samples from 289 water sources used by 238 utilities. Of the 256 ground sources, 51 are not chlorinated, and these are found exclusively in small towns. Although surface sources represent but 11% of the total number of sources, they serve 23% of the population using community supplies. Almost 2 million Iowa residents are served by community supplies serving 1,000 people or more, and the remaining million residents use water from very small community sources or from their own private wells.

Chloroform Dose and Chloroform Levels

Figures 2–6 show data on chloroform levels and chlorine dosage in the ground and surface sources of Iowa. Levels of nonvolatile chlorinated compounds of higher molecular weight tend to vary with chloroform levels, so chloroform measures may be interpreted as representing a much larger class of compounds, many with demonstrated mutagenic and carcinogenic properties.

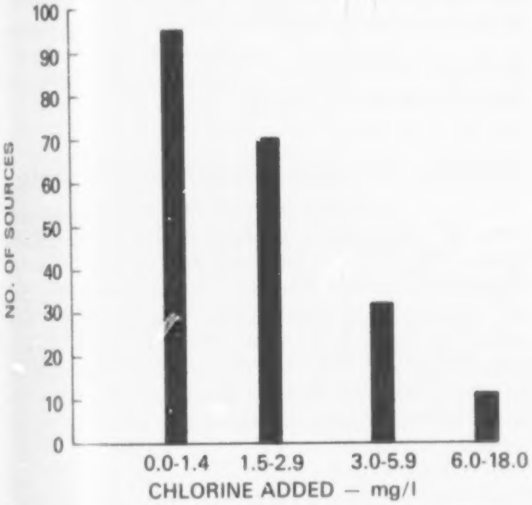

FIGURE 2. Distribution of annual average chlorine dose among ground water sources, Iowa, 1979.

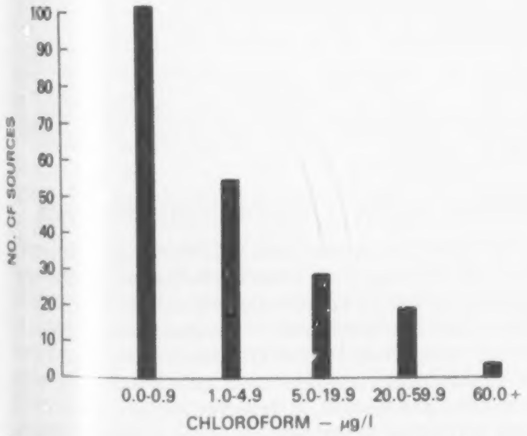

FIGURE 3. Distribution of measured chloroform levels in finished water from chlorinated ground sources, Iowa, 1979.

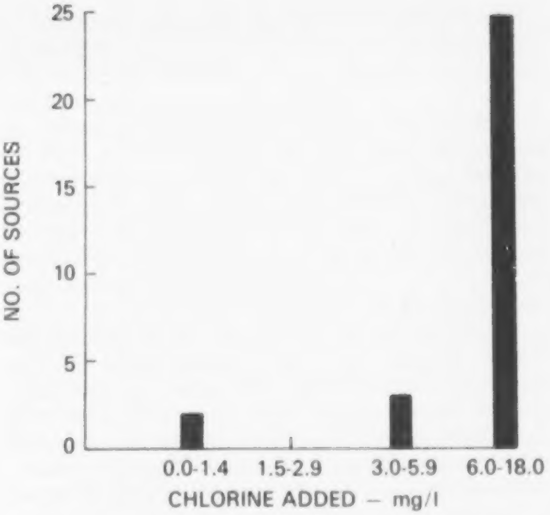

FIGURE 4. Distribution of annual average chlorine dose among surface water sources, Iowa, 1979.

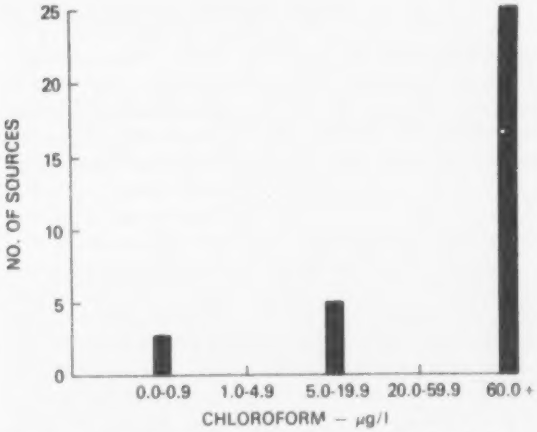

FIGURE 5. Distribution of measured chloroform levels in finished water from chlorinated surface sources, Iowa, 1979.

Figure 2 shows how many ground sources had chlorine dosages in given ranges. It demonstrates that most ground sources were treated with less than 3 mg/l., with relatively few ground sources in the higher dose ranges.

Figure 3 shows the number of these same ground sources with measured chloroform levels falling in different ranges. The distribution pattern of chloroform levels parallels the chlorine dosage pattern. Three-fourths of the samples from ground sources had chloroform levels of less than 5 μg/l.

The distribution among surface sources of chlorine dosage and measured chloroform levels provides a sharp contrast with the distribution among ground sources. Figure 4 shows how many surface sources had chlorine dosages of given levels. In contrast to ground sources, the chlorine dose in most surface sources was greater than 6 mg/l. Most U.S. water supplies observe breakpoint chlorination. That is, chlorine is added until a free residual is produced. If the raw water contains high levels of organics, more chlorine is necessary to oxidize materials before free chlorine is available.

Figure 5 shows chloroform levels among the surface sources. Surface sources tended to have elevated levels of chloroform, in contrast with ground sources, with most measuring above 60 μg/l. Half of the 33 surface sources in Iowa had chloroform levels greater than 107 μg/l.

Figure 6 shows the distribution of chloroform as a function of annual average chlorine added, in surface sources and in ground sources. Levels of both chloroform and of added chlorine are elevated in surface sources, as mentioned above. Within the surface or ground source category, however, there is no within-group association of added chlorine with measured chloroform level. This counterintuitive observation may result from the types of data we collected. "Chlorine added" is the reported average dose over the year prior to sampling, and chloroform was measured in a one-time grab sample in which the haloform reaction was allowed to go to completion. The lack of correlation between chlorine dose, and measured chloroform level, within source groups, implies that records of historical chlorine dose may not provide reliable and acceptable guides to levels of some classes of chlorination by-products in finished drinking waters. It is clear, though, that there are large differences in chlorine dose and in chloroform level (and presumably in levels of nonvolatile chlorinated compounds) between ground and surface sources. Historical dosage information would appear to provide no better a guide to the mutagenic or carcinogenic potential of organics in finished drinking water than a categorical definition of source type, such as "surface" or "ground". Evaluation of data from the other nine study areas will help clarify this issue.

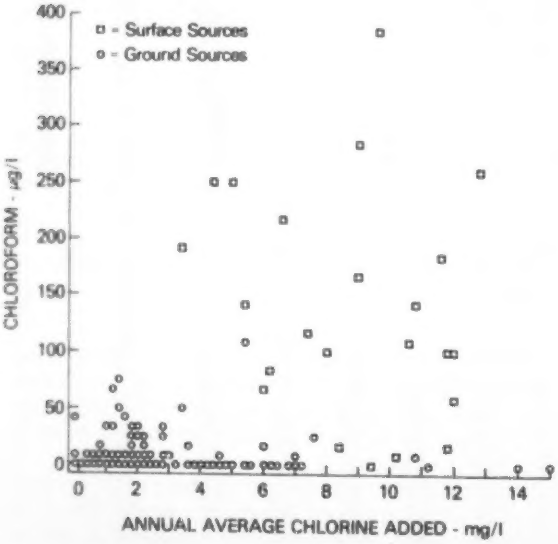

FIGURE 6. Measured chloroform levels versus chlorine dose in finished water from surface and ground sources, Iowa, 1979. Many of the points represent 2 or more measurements.

Review of Epidemiologic Studies

Since 1974, at least 29 epidemiologic studies have assessed the relation between one or more aspects of water quality and one or more forms of cancer. These studies differ markedly in design and in what they can reveal about possible water-cancer relationships. Several reviews of these studies have been prepared (*10–14*) and these will not be repeated here. The studies may be evaluated from several points of view including the general study design (ecologic or case-control), the choice of health endpoint used as a measure of effect, the use and availability of other exposure factors or host characteristics which may confound associations, and the choice of water exposure variables or surrogate variables. One major distinction to be made among the studies is between geographic correlation and case-control studies. Another distinction is between studies based on mortality measures and those based on morbidity. The great majority of completed studies are correlational studies based on mortality rates, usually aggregated on a county level.

Geographic Correlation Studies

Geographic correlational studies usually use existing data resources, and therefore may be performed rather inexpensively and rapidly. The results from such studies can serve as qualitative guides to potential environmental risk factors and the types of health impact which they may have. Following the first perception of possible cancer risks from contaminated drinking water, a number of geographic correlation studies were mounted. Most used multivariate techniques to seek associations between county cancer mortality rates and one or more surrogate measures of water quality from the predominant county drinking water supply. In most, a multiple regression model was used to adjust for county demographic and industrial characteristics known or thought to be related to the malignancy under study. The dependent variable in each model was a rate, specific for sex and race. Calculated values of the regression coefficients estimate the relative contributions of the "independent" variables to the cancer rate.

Three geographic correlation studies provide examples of the different types of surrogate exposure variables that have been used. Page and co-workers (*15*) addressed the issue of whether county parish populations in Louisiana served by the Mississippi had higher cancer rates than other parishes. The water exposure variable was the percentage of a parish's population using Mississippi water, and it was included in the regression model along with several variables for parish industrial and socioeconomic characteristics.

Salg (*16*) examined data from the 346 counties of the Ohio river drainage basin. The exposure index in one analysis was the percent of a county's population served by surface water, and in another, the percent with pre-chlorinated water. Each county cancer mortality rate, specific for sex and race, was regressed on the water exposure variable and several county demographic and industrial variables.

Cantor et al. (*17*) looked at cancer rates in counties with water supplies sampled by the Environmental Protection Agency in 1975. The multivariate analyses were restricted to the 76 counties with more than 50% of their population served by the sampled supply. The exposure was one of three continuous variables: the total trihalomethane level; the chloroform concentration; or the brominated trihalomethane level. Several demographic, socioeconomic, and industrial characteristics of the study counties were included in the regression model.

The primary purpose of the correlational study is hypothesis generation; that is, a preliminary evaluation to determine if more detailed studies might be warranted. Inferences from correlational studies are limited because information on such potentially important factors as migration and historical exposure patterns is often incomplete or nonexistent. The measure of effect is the county cancer rate, a group characteristic, and not the particular experience of individual group members. Individuals who die of cancer may not be the most highly exposed members of the population. Information is often missing on other factors related to the risk of disease, such as cigarette smoking, and an observed association may be due to the confounding influence of these uncontrolled variables. Positive associations from such studies must therefore be interpreted with caution since they may not reflect a cause-effect relationship.

The suspicion that a causal relationship may underly statistical associations is increased, however, if several independent studies observe similar patterns of correlation in different regions, among different populations, and in both sexes. A report by the National Academy of Sciences (*10*) and more recent reviews by Wilkins et al. (*11*) and by Shy and Struba (*12*) are in general agreement that results from ecologic and case-control studies point to possible associations for bladder, rectal, and colon cancer. In a review completed in 1978, we noted consistencies in geographic associations between bladder cancer and drinking water quality (*13*). Among the eleven studies we reviewed, eight revealed positive and statistically significant associations among white males between bladder cancer rates and one or another measure of drinking water contamination.

Case-Control Studies

Case-control studies, which use information on individuals, can provide quantitative estimates of risk. The basic approach is to gather personal histories for persons with the disease of interest and for a series of matched individuals without the condition (controls). Estimates of risk derive from comparisons of the exposure of cases and controls. The case-control studies released to date are based on mortality records. These studies represent a major advance over ecologic studies. The drawbacks which remain derive in large part from limitations of the primary data source they use: the death certificate. Characterization of drinking water source is based on a link of water supply data to the address on the death certificate, and thus represents the last drinking water source of the decedent. Misclassification of exposure can occur if the individual had recently migrated from an area with different drinking water source characteristics. Another weakness of case-control studies based on death certificates is their inability to adjust for other exposures which may have caused the disease. The investigator is not able to control for diet or cigarette smoking, and the only occupational information available is that listed on the certificate of death. Despite these weaknesses, the case-control studies based on death certificates are a major methodologic improvement over the earlier ecologic studies, and they have provided a significant contribution to our understanding of drinking water quality and cancer.

The Council on Environmental Quality recently released a contractor's report in which five case-control death certificate studies were reviewed (*14*). The reviewers noted the consistency among the studies and positive associations between rectal, colon, and bladder cancers, and one or more water quality variables. They noted that associations with rectal cancer were observed in all five studies.

Case-Control Interview Studies: An Example

An alternative to the case-control study based on death certificates is the interview study that uses information collected directly from patients and their matched controls. At least five such studies are currently in progress (Table 1). Each is examining the association of many risk factors, among them water quality factors, with one type of cancer. The anatomic sites in these ongoing studies are those that were suggested by the completed studies: colon, rectum, and bladder. These case-control interview studies do not suffer from many of the drawbacks of mortality studies.

The suggested links between drinking water quality and bladder cancer are being pursued by incorporating a water quality component into a large case-control interview study of bladder cancer. This investigation was originally motivated by the need for information on saccharin. The case-control design permitted, and indeed required, that information on other known and suspect causes of the disease also be gathered and considered in the analysis. In this study, we interviewed 3000 persons who were newly diagnosed with bladder cancer during 1978 in five states and five metropolitan areas of the United States, and a series of 6000 controls. Age was restricted to the range 21–84. Controls aged 21–64 were chosen using a random digit dialing method. Older controls were randomly sampled from files of the Health Care Financing Agency. By personal interview, we obtained information on demographic background, smoking history, occupational history, relevant medical information and a history of artificial sweetener use. Information on several other items such as coffee drinking habits, use of hair dyes and fluid ingestion patterns were also gathered. A report on saccharin has been released (*18*).

Information from a residential and water supply history for each respondent is being used to create a lifelong water quality profile. We obtained a lifetime residential history from each participant and asked whether the primary drinking water source at each residence was community, private well, bottled, or other. Independently, we gathered data from more than 1000 public utilities in the ten study areas. We collected historical information on the raw water sources, the treatment practices, and the geographic areas served by each purveyor, going as far back as 1900. Information on water source includes whether the source is surface, ground or purchased. Surface waters are further characterized by the amount and type of potential contamination. Ground sources are characterized by the depth and number of wells and characteristics of the aquifer. If the major source of water was another water supply, the supplier is indicated and a link is then made to his records. Many types of treatment information are recorded, including mechanical treatment, chemical treatment other than chlorination, and chlorination. An attempt was made to retrieve information on chlorine dose, but in our experience, this information is often not available. A list of the geographic areas served is also collected. Some data was obtained from centralized state or federal records. In all cases, it was also necessary to visit the utilities and obtain information directly from their records or from personal interviews with utility officials.

Table 1. Case-control studies in progress.

Investigator(s) (inst./funding)	Anatomic sites	Cases/ controls	Locale	Sex/race	Completion date	Design notes
Cantor et al. (NCI/EPA/FDA)	Urinary bladder	3000/6000	10 Areas, U.S.	All	1982	Population based interview
Gottlieb (Tulane/EPA)	Colon, rectum	500/500	SE Louisiana	All	1984	Hospital based interview
Kanarek and Young (U. Wisc./EPA)	Colon	200/400	Wisconsin	WM, WF	1983/4	Population based interview
Shy and Struba (UNC/NIH)	Colon	300/600	N. Carolina	All	1982	Hospital based interview
Trock (N.Y. State)	Colon, rectum	400/400	N.Y. state (Outside N.Y.C.)	F	1982	Mortality records, confirmed diagnoses, 20 yr residential history

The potential value of these data for the bladder cancer case-control study was estimated by calculating the total years of experience spent in and out of study areas by participants and seeing for how many years they were served by a municipal source, a private well or spring, or by some other source. We were able to account for approximately 90% of the estimated total person-years of all cases and controls. Of these, 342,844 person years (62.8%) were spent within study areas on municipal supplies where we have excellent coverage of historical community supply information; 14.3% of respondent person-years were spent in areas with municipal supplies where we have limited information, or on other water sources; 109,295 respondent person-years (20.0%) were at residences where a private well or spring was the major source of drinking water. The total number of person-years spent in residences with private wells or springs or with a municipal source of water where we have the possibility of getting specific information represents approximately 452,000 person-years of experience or 75% (83% × 0.90) of the total lifetime experience of all cases and controls. This level of exposure ascertainment is well within acceptable bounds for epidemiologic evaluation.

High-speed digital computers allow the merging of historical community water supply information with the personal residential histories of study respondents. For each year, the complete record of each individual contains information on the water source type from the interview, and if the respondent indicated that a community source was used, detailed information on that drinking water source. In the first epidemiologic analyses, we are looking at just a few features of drinking water source, namely, whether the water is chlorinated or not and whether the water comes from a surface or a ground source. It is possible, of course, to return to the original utility record to pull in any additional information which is deemed important.

The types of records being created are demonstrated in Figures 7–9, which describe historical types of water source serving study participants from Iowa. Three source categories are shown: ground not chlorinated; ground chlorinated; and surface chlorinated. Figure 7 shows, for respondents who had a nonchlorinated ground source in 1975, the type of source that they had from 1910 onwards. It suggests that people on this type of source have had limited residential mobility. If exposures of importance had occurred prior to 1950, use of the 1975 drinking water source to characterize earlier exposures in an epidemiologic study would not result in important misclassification among this group. In contrast are the people in Iowa presently consuming chlorinated water. Figure 8 shows the distribution over time of the 364 respondents whose homes were served by chlorinated ground sources in 1975. Prior to 1950, fewer than half of this group were served by the same kind of water. The increase in the use of chlorinated ground water represents a shift from nonchlorinated ground water among this population. It is likely that this shift represents the increasing practice of chlorination in modest sized communities with ground sources. The use of the 1975 source definition in epidemiologic studies could lead to misclassification of exposure in this group of individuals. Figure 9 shows similar data for the 231 respondents whose source of drinking water in 1975 was a surface chlorinated source. Before 1955, less than 40% of this group were drinking chlorinated surface water. A fourth category, "other" represents the percentage that each year's total is less than 100% In the "other" category are people who reported a municipal drinking water source from outside of the study

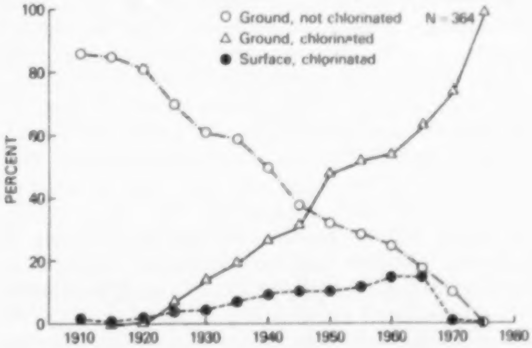

FIGURE 7. Type of water source, 1910–1975, of Iowa study respondents with a nonchlorinated ground source in 1975.

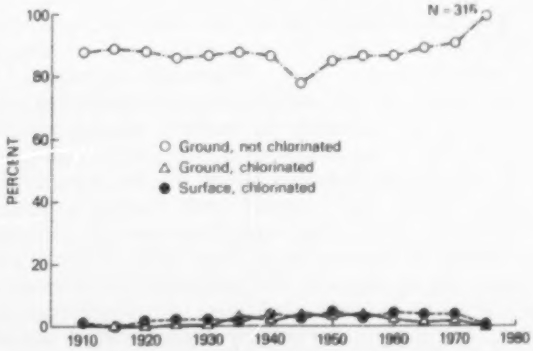

FIGURE 8. Type of water source, 1910–1975, of Iowa study respondents with a chlorinated ground source in 1975.

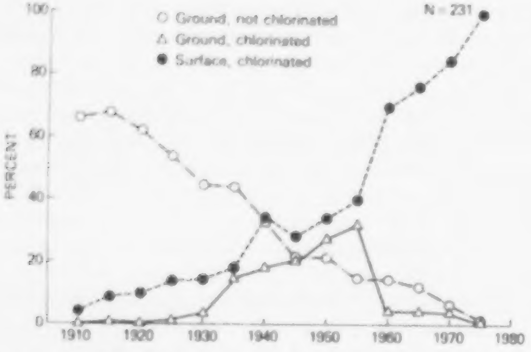

FIGURE 9. Type of water source, 1910–1975, of Iowa study respondents with a chlorinated surface source in 1975.

area, or who were among the number who had some unusual source or did not report a drinking water source for the year in question. Estimates of risk based on the 1975 water source in an epidemiologic study would likely underestimate the real risk, because the greatest shifts have been from ground, nonchlorinated to the chlorinated categories. We do not know at this time how representative the experience of the Iowa study population may be of the places where ecologic or case-control death certificate studies of water quality have been performed. These data suggest that the completed ecologic and case-control studies based on mortality data alone may have underestimated effects because of misclassification of exposure. Studies that accounted for changes in water source due to migration or to modifications in water treatment such as the work of Young (*19*) and of Gottlieb (*20*) may not be so seriously affected by the misclassification problem as some of the others.

Summary and Conclusion

The major strengths of the case-control interview study design in the evaluation of water contaminants are many. Historical exposures of individuals may be defined with a completeness and accuracy not possible in ecologic studies or in case-control studies based on mortality records.

Collection of information on a wide variety of other known and suspect risk factors permits control for these other exposures as well as measurement of interaction of them with water exposures. Let us suppose that products of chlorination did not influence the likelihood of bladder cancer, but that people served by contaminated water smoked at higher rates than people served by pristine sources. An analysis that could not adjust for smoking might mistakenly associate bladder cancer with poor water quality. Alternatively, if water-borne carcinogens were causally related to the disease and smoking data were available, then elevated risks for exposures to water contaminants would be seen among smokers alone, and among nonsmokers alone. This would also be true of persons with and without exposures to occupational carcinogens and other exposures.

The wealth of historical exposure information in a case-control interview study permits evaluation of dose-response relationships. A causal interpretation of an association is strengthened by data showing that the risk of individuals increases with the number of years exposed. The time from first exposure to a presumed carcinogenic factor to the appearance of an adverse effect also may be evaluated, and can provide important confirmatory evidence.

We are now on the threshold of obtaining results from carefully conducted case-control interview studies of bladder, colon and rectal cancers which will provide quantitative estimates of risk related to products of chlorination and other water contaminants. Our preliminary evaluation of historical changes in individual water sources suggests that the estimates of risk from the completed case-control mortality studies are conservative, and that the real risks may be much greater. There remains a need for further confirmatory evidence from toxicologic evaluation of the volatile and nonvolatile products of chlorination found in drinking water. Laboratory research which has combined chemical fractionation of bioactive residues isolated from drinking water, combined with *in vitro* biologic assays, is a powerful tool in this regard. The mounting evidence linking chlorination products in community drinking water supplies with elevated risk of human cancer poses a dilemma and a challenge. Quantitative estimates of risk from epidemiologic studies soon to be available will provide a firm and defensible factual basis that can assist in making the challenging decisions of whether or not to further control levels of chlorination by-products and other organic contaminants of public drinking waters.

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Environmental Health Perspectives
Vol. 46, pp. 197-205, 1982

Mutagenic Activity Associated with By-Products of Drinking Water Disinfection by Chlorine, Chlorine Dioxide, Ozone and UV-Irradiation

by B. C. J. Zoeteman,* J. Hrubec,* E. de Greef* and H. J. Kool*

A retrospective epidemiological study in The Netherlands showed a statistical association between chlorination by-products in drinking water and cancer of the esophagus and stomach for males. A pilot-plant study with alternative disinfectants was carried out with stored water of the Rivers Rhine and Meuse. It was demonstrated that the increase of direct acting mutagens after treatment with chlorine dioxide is similar to the effect of chlorination. Ozonation of Rhine water reduced the mutagenic activity for *Salmonella typhimurium* TA 98 both with and without metabolic activation. UV alone hardly affects the mutagenicity of the stored river water for *S. typh.* TA 98. In all studies, practically no mutagenic activity for *S. typh.* TA 100 was found. Although remarkable changes in the concentration of individual organic compounds are reported, the identity of the mutagens detected is yet unclear. Compounds of possible interest due to their removal by ozonation are 1,3,3–trimethyloxindole, dicyclopentadiene and several alkylquinolines. Compounds which might be responsible for the increased mutagenicity after chlorination are two brominated acetonitriles and tri(2–chlorethyl) phosphate. Furthermore, the concentration procedure with adsorption on XAD resin and the subsequent elution step may have affected the results. It is proposed to focus further research more on the less volatile by-products of disinfection than on the trihalomethanes.

Introduction

In The Netherlands most of the drinking water does not receive a chlorine treatment. The bulk of the use of chlorine is related to surface water treatment. Even part of the drinking water derived from polluted surface water is distributed without chlorination which often is the case when bank-filtration or dune infiltration is applied. From the total chlorine consumption of 2100 tons by the waterworks of The Netherlands in 1977, 800 tons were used for raw water transport, 600 tons for break point chlorination and 700 tons for final disinfection (*1*). Since then chlorine application has been reduced as much as possible. Alternatives for chlorination which have equal oxidation or disinfection potential and which create smaller health risks are investigated by the National Institute for Water Supply (NIWS) (*2*).

The health effects of chlorination itself were studied in more detail by the NIWS. An epidemiological study was carried out, as part of a larger epidemiological study in the European Communities, under contract 273-77-1 ENV N with the Commission of the European Communities. This study is presently finalized and includes a population of 4.6 million inhabitants of the Netherlands for which cancer mortality rates for both sexes and seven cancer sites were obtained from the Central Bureau of Statistics over the years 1965–1976. The standardized mortality rates for the age group 35–64 (Table 1) showed a statistical association

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Table 1. Correlation coefficients between standard cancer mortality ratios in both sexes and drinking water quality in the Netherlands.

	Water quality aspect					
	Ground water/surface water		No chlorination/chlorination		Level of THM[a]	
Type of cancer	Male	Female	Male	Female	Male	Female
Esophagus	−0.02	0.18	0.27	0.19	0.60[b]	0.29
Stomach	0.04	−0.13	0.36	0.15	0.46[b]	0.27
Colon	−0.01	−0.12	0.00	−0.19	−0.18	−0.24
Rectum	−0.23	0.02	−0.08	0.38	−0.04	0.22
Liver	0.39[b]	0.11	0.56[b]	0.14	0.07	0.16
Bladder	0.39[b]	−0.13	0.17	−0.12	−0.03	0.03
Lung	0.53[b]	0.43	0.52[b]	0.32	0.25	−0.14

[a] r is significant ($p < 0.05$, one-tailed test).

[b] THM data obtained from a survey carried out in 1976 (*3*).

between chlorination by-products, as indicated by the trihalomethanes (THMs) and cancer of the esophagus and the stomach in males. Liver cancer and bladder cancer were not associated with chlorination by-products, although for males these cancer sites showed an association with the use of contaminated surface water as a raw water source. A further correlation study with organic micropollutants detected in drinking water derived from surface water (*3*) suggests an association between liver cancer and chlorobenzenes for males ($r = 0.47$), stomach and rectum cancer and alkylbenzenes for females ($r = 0.57$ and 0.59, respectively) and bladder cancer and phthalates for males ($r = 0.39$). However, as indicated by the lung cancer data, the areas supplied from contaminated surface water are situated in the most industrialized part of the country, which may confound the issue substantially (*4*).

A study of potential health hazards of alternative disinfectants for chlorine is being carried out by the National Institute for Water Supply in a small-scale pilot plant at Dordrecht as described earlier by De Greef et al. (*2*). Disinfection methods including ozone, chlorine dioxide and UV are studied in comparison with chlorine. The mutagenic activity of stored water of the Rivers Rhine and Meuse is examined after application of these agents. Furthermore, an attempt has been made to identify compounds which may be partly responsible for the mutagenicity observed in the Ames test applied. This paper presents the data obtained so far with the pilot installation.

Materials and Methods

Pilot Plant

The schematic flow diagram of the pilot plant is shown in Figure 1. Raw material is supplied to the pilot plant in a mobile zinc coated steel tank of 2 m^3 from which it is pumped at a rate of 0.270 m^3/hr into the disinfection unit which consists of two glass tube reactors. The reactors are 2 m high and each reactor has a volume of 64 liters. The residual time in the reactor is about 15 min.

Aqueous solutions of sodium hypochlorite or chlorine dioxide are dosed into the water supply line of the first reactor. Prior to the disinfectant dosing pH adjustment takes place by means of automatic dosing of hydrochloric acid or sodium hydroxide.

Ozone gas is injected through ceramic diffusers at the bottom of the reactors. Ultraviolet irradiation takes place in the sterilizer located behind the reactors. The sterilizer can be directly connected with the mobile tank by means of a bypass.

In some experiments the disinfected water was treated by direct filtration with addition of ferric chloride (10 mg Fe/l.) in a double layer filter (filtration rate of 3.5 m/hr) and by activated carbon filtration (filtration rate 9 m/hr, contact time 7 min).

Chlorine dioxide was generated by the sodium chlorine acid activation technique to minimize the simultaneous formation of free chlorine (*5*). A commercially available generator (Bello-Zon, Filt[illegible] GmbH, Heidelberg) to produce a stock solution of chlorine dioxide by mixing of sodium chlorite solution (7%) and hydrochloric acid (9%) was used. The concentration of free chlorine in the produced ClO_2 solution did not exceed 6%.

Ozone was produced with a Trailigaz air-fed unit, model Labo 76, with a production capacity of 3-9 g/hr.

Ultraviolet disinfection took place with a BEL-UVA-D1 unit (Berson Milieutechniek, Nuenen, The Netherlands) consisting of a stainless steel cylindrical chamber, housing a longitudinally mounted 30-watt ultraviolet lamp. The radiation intensity during the experiments was high (120 mW-sec/cm^2) at a detention time in the unit of 26 sec.

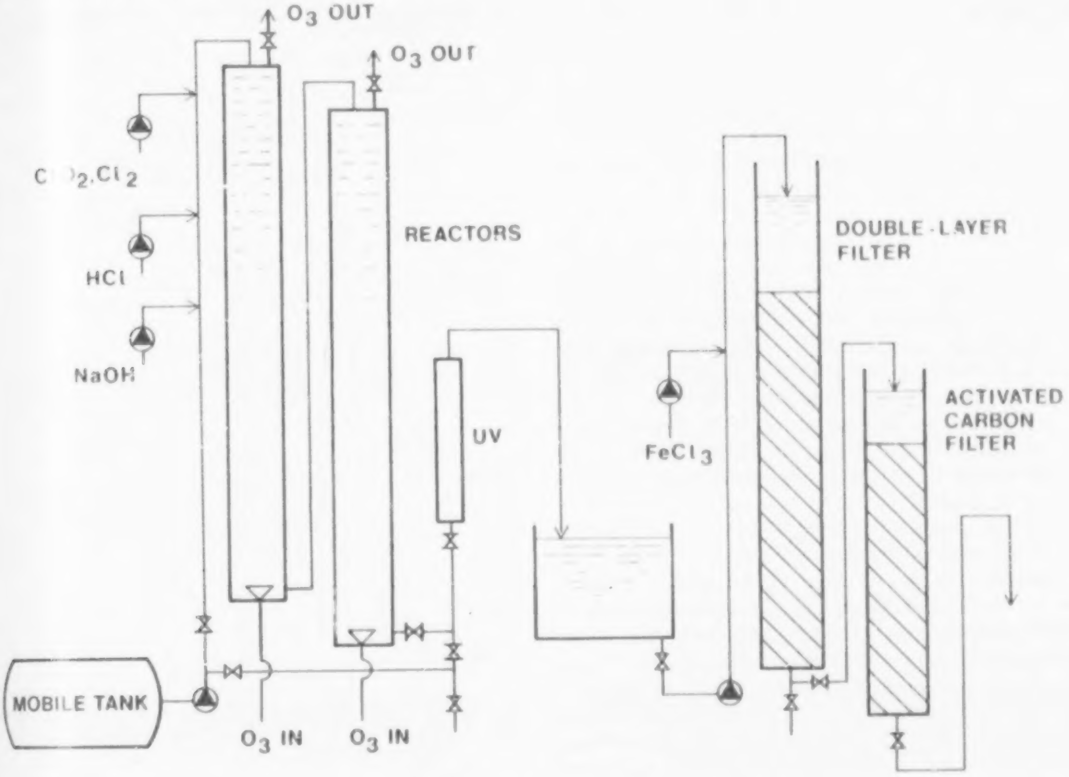

FIGURE 1. Flow diagram of the pilot plant at Dordrecht.

All e periments were monitored by standard microbio ogical testing for the effective removal of microor anisms. In all cases microbiological standards fo drinking water were met.

Chemi al Analyses

Resid al concentrations of chlorine and chlorine dioxide water were measured by the DPD-FAS techniq (*6*). Chlorine, chlorine dioxide and chlorite in tock solution were determined by the iodomet c method described by Berndt (*7*) and Valenta nd Gabler (*8*).

Resid al concentrations of ozone in water were measur iodometrically (*9*). Ozone concentration in the i et and exhaust gas was determined iodometrical after passing a sampled gas stream through gas-was ing bottles containing a 2% potassium iodide solution

For direct headspace analysis of very volatile halogen ted compounds the procedure described by Piet et . (*10*) was used.

Ident fication of organic compounds in XAD-ether concentrates was carried out according to a procedure developed by Morra et al. (*11*) using a Finnigan 4000 masspectrometer coupled with an INCOS datasystem.

Mutagenicity Testing

Mutagenicity was tested according to the method developed by Ames et al. (*12*) using *Salmonella typhimurium* (TA 98 and TA 100) with and without metabolic activation with S9–mix. The organic substances in the water had to be concentrated 2000–4000 fold from 40–80 liters of water, which was carried out by adsorption on XAD-4/8 resin followed by DMSO extraction. A volume of 0.25 ml and 0.5 ml of the DMSO concentrate was directly tested in the Ames test as described by Van Kreijl et al. (*13*) and Kool et al. (*14*) in order to obtain dose-response curves for the samples tested. In most cases no significant increases in mutagenic activity and dose response were observed with the TA 100 strain with and without metabolic activation. Therefore only the results obtained with the TA 98 strain are reported in this paper.

Results

Chlorine

According to the results of a number of chlorination experiments with stored Rhine water and stored Meuse water, chlorination tends to increase the mutagenic activity as shown in Figure 2.

Figure 2 illustrates that the effect of chlorination on the mutagenic activity depends strongly on the type of organic compounds present in the water. The increase in activity from direct acting mutagens due to chlorination is much more pronounced for Meuse water than for Rhine water. With metabolic activation chlorination of Rhine water can result in a reduction of the mutagenic activity. This is best explained by supposing that Rhine water contains more indirect mutagenic compounds than Meuse water, although other possible explanations cannot be excluded.

Due to the testing procedure applied, the volatile THMs formed by chlorination are not likely to be responsible for the mutagenicity found. The identity of the less volatile chlorination by-products which are probably responsible for the increased mutagenicity detected is yet unclear. GC-MC analysis of the XAD-ether extract containing the compounds formed resulted in the identification of a few substances, which are listed in Table 2. It should be noted that the ether-extract generally contained less mutagenic activity than the DMSO extract. Of particular interest are the two identified halogenated acetonitriles and tri(2-chloroethyl) phosphate. According to Nakamura et al. *(15)* the latter compound is a weak mutagen. Further studies using HPLC techniques are continuing in order to identify the compounds formed which may contribute to the increased mutagenic activity of chlorinated water.

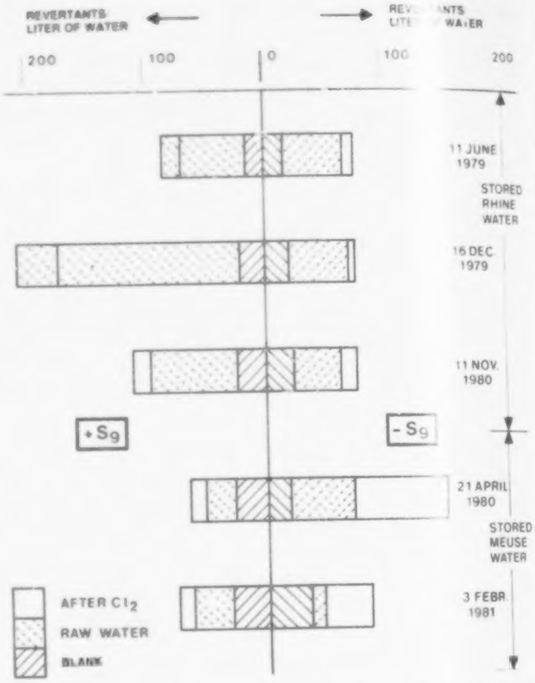

FIGURE 2. Effect of chlorination (5 mg Cl_2/l.) on mutagenicity of XAD-DMSO concentrates tested with *S. typhimurium* TA 98 (pH 8–9).

Chlorine Dioxide

Oxidation with chlorine dioxide generally resulted in a significant increase of the activity due to direct acting mutagens (Fig. 3). After metabolic activation, a considerable reduction of the mutagenic activity was observed in the case of chlorine dioxide application to Rhine water.

Table 2. Substances formed by chlorination of stored Rhine water (dose 5 mg Cl_2/l.; pH 8.1; 6.5°C).

Retention index	Compound	Concentratio[illegible]g/l.	
		Before	A[illegible]r
250	Chloroform	0.1	[illegible]
370	Bromodichloromethane	0.1	[illegible]
600	Dibromochloroacetonitrile	0.5	[illegible]
675	Bromochloroacetonitrile	—[a]	[illegible]
875	Bromoform	—	[illegible]
930	Dibromoacetonitrile	—	[illegible]
1200	Acetopheone	—	[illegible]
1370	Unknown alcohol	—	[illegible]
1390	Bis(2-chloroisopropyl) ether	0.3	[illegible]
2195	Bromodichloropropane	—	[illegible]
2562	Unknown oxygenated compound	—	[illegible]
2670	Tri(2-chloroethyl) phosphate	1	[illegible]

[a] Denotes less than 0.1 μg/l.

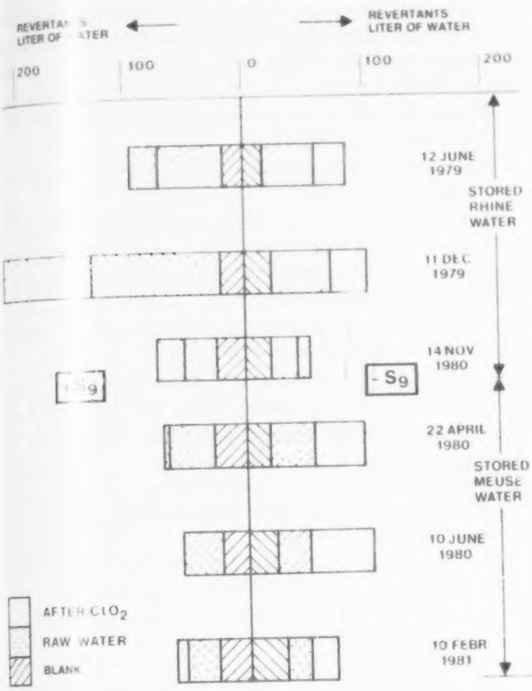

FIGURE 3. Effect of chlorine dioxide (5 mg ClO_2/l.) on mutagenicity of XAD-DMSO concentrates tested with *S. typhimurium* TA 98 (pH 8–9).

In general it seems that chlorine dioxide evoked similar or even more pronounced effects on the mutagenic activity of water than those found for chlorine. Furthermore, the effect of both oxidants is reasonably reproducible for a specific water source. During the ClO_2 experiments no important quantities of volatile halogenated compounds were formed. The THMs were generally not present at levels above 0.1 g/l. GC-MS data from previous tests ( ) suggested that e.g. 2.6-di-*tert*-butyl-4-aminomethylphenol, 2,2,4-trimethylpenta-1,3-diol diisobutyrate and 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde are formed. The type of changes in mutagenicity indicates that the mutagenic compounds detected may be similar to those involved in chlorination processes.

Additional experiments, shown in Figure 4, indicated that the mutagenic substances originating from the raw water source or from chlorine dioxide oxidation can be effectively removed by coagulation/filtration subsequently followed by carbon filtration.

A similar positive effect of iron coagulation on the reduction of the mutagenic activity was reported by Denkhaus et al. (*16*).

Removal of mutagens by activated carbon filtration was described also by Kool et al. (*14*) and Hrubec (*17*). However, others have found activated carbon to be inefficient in reducing mutagenic activity (*16,18,19*).

Possibly the use of "old" and "fresh" activated carbon provides an explanation for these contradictory findings.

Ozone and UV

Among the disinfectants, ozone and ultraviolet irradiation are two extremes. Ultraviolet radiation is expected to affect the identity of the organic substances only to a minor extent while ozone will oxidize organic compounds more strongly than chlorine and chlorine dioxide. In accordance with these characteristics mutagenic activity of water is unaffected by ultraviolet treatment, while the high oxidation potential of ozone results in a reduction of the mutagenicity detected in these experiments both with and without metabolic activation, as shown in Figure 5.

Similar results for ozone applied in wastewater treatment were reported by Denkhaus et al. (*16*)

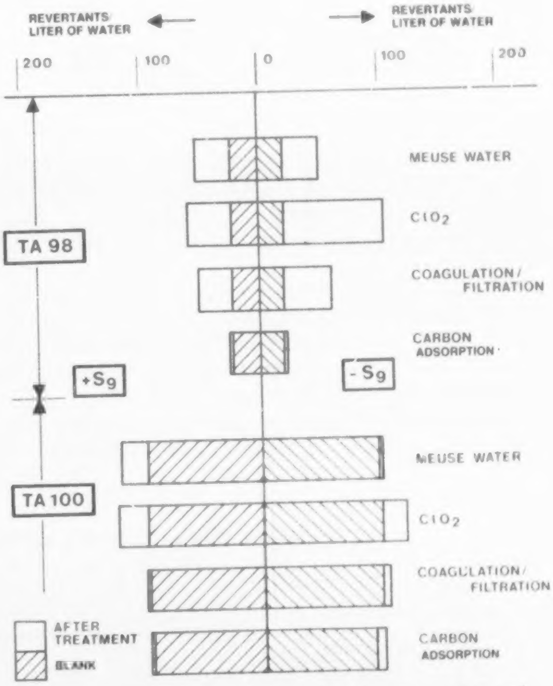

FIGURE 4. Effect of chlorine dioxide (2 mg/l.) and successive treatment by coagulation/filtration and carbon adsorption on mutagenicity of Meuse water concentrates (XAD-DMSO, *S. typhimurium* TA 98 and TA 100).

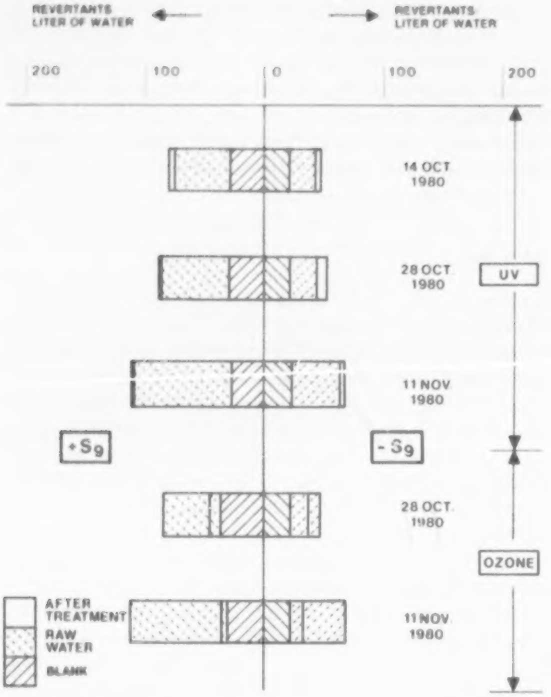

FIGURE 5. Effect of ultraviolet irradiation and ozonation on mutagenicity of stored Rhine water (XAD-DMSO, *S. typhimurium* TA 98).

and Hrubec (*17*). Also Ishizaki et al. (*20*) demonstrated detoxification of pollutants by ozonation. Indications of increases in mutagenicity after ozonation were reported by Gruener (*21*) and Simmon et al. (*22*).

GC-MS analysis of the compounds formed by ultraviolet and ozone treatment was up till now not very successful in identifying the compounds concerned. As Tables 3 and 4 indicate, oxygenated aldehydes and ketones at levels of 0.1–3 g/l. were detected in the ozonated water while the ultraviolet treatment seemed to result in the formation of benzene, toluene and compounds closely related to the substances present in the raw water such as a dihydrotrimethylquinone isomer and a reaction product of 3,3-dimethyl-1,5-bis(isobutyl) bicyclo(3,1,0)-hexanone-2, which is presumably formed in the storage reservoir by algae.

The combination of ozone and ultraviolet irradiation was also studied.

In summary, it can be concluded that UV irradiation does not affect mutagenicity of Rhine water significantly, while ozonation reduces the mutagenic activity considerably both with and without metabolic activation. Furthermore, no formation of lower halogenated compounds was observed.

Comparison of Chlorine and Its Alternatives

The main question remaining after the discussion of the alternate disinfectants is to what extent the alternatives are better then chlorine from a health point of view. Comparative mutagenicity data for all disinfectants, including the combinations of ClO_2 with UV and O_3 with UV have been obtained for stored Rhine water and are presented in Figure 6. From these data it is clear that the mutagenicity detected of the by-products of chlorine dioxide with and without a combined ultraviolet treatment is similar to the mutagenicity detected for chlorination by-products. In this respect, the large difference in formation of trihalomethanes under these conditions seems not to be indicative for the associated health risks. However, ozone provides, at least in the case of Rhine water treatment, a better alternative to chlorine as far as the mutagenicity of the detected by-products is concerned. Both the mutagenicity of the water with and without metabolic activation is reduced. This effect is even more marked for the combination of ozone with ultraviolet irradiation. Ultraviolet treatment alone hardly affects the mutagenicity of the water.

In accordance with these observations are the GC-MS data which indicate the compounds which

Table 3. Substances formed by ozonation (8 mg/l.) of stored Rhine water.

Retention index	Compound	Concentration, g/l.	
		Before	After
980	Heptanal	—	1
1275	Unknown oxygenated compound	—	0.1
1700	Unknown oxygenated compound	—	0.1
1750	Unknown oxygenated compound	—	0.3
2490	Unknown aldehyde	—	0.3
2555	Unknown oxygenated compound	—	1
2855	Unknown ketone	—	3

Tabl 4. Substances formed by ultraviolet irradiation (120 mW-sec/cm^2) of stored Rhine water.

etenti index	Compound	Concentration, µg/l. Before	Concentration, µg/l. After
585	Benzene	0.1	1
550	Toluene	—	3
2210	Dihydrotrimethylquinoline isomer	—	1
2320	Unknown	—	10
2515	Unknown[a]	—	1
2580	Unknown	—	1

ere reduced in concentration by the alternate isinfection techniques. Table 5 illustrates that more ompounds are eliminated where the more powerl oxidants are applied. Ultraviolet treatment hardly ffects the concentration of the compounds identified, hile ozone removes nearly all substances listed. ome of the compounds given in Table 5 such as the uinolines (*23*), might contribute to the mutagenic ctivity detected in Rhine water. However, the ubstances present in the XAD-ether concentrate an not be directly linked to the mutagenicity of the AD-DMSO concentrates as the latter were shown o contain generally a higher mutagenic activity. Infortunately the DMSO concentrates are not suitble for direct GC-MS analysis. A further exploraion of this procedure aiming at the identification of nore polar disinfection by-products is presently ndertaken.

Other complications must be envisaged too. It till cannot be stated for certain whether the changes bserved in mutagenic activity are really related to hanges in mutagenicity of the water or if these are influenced by a different adsorption behavior of the mutagens on the XAD. The latter can result simply from the altered chemical properties of chemicals present after the disinfection treatment.

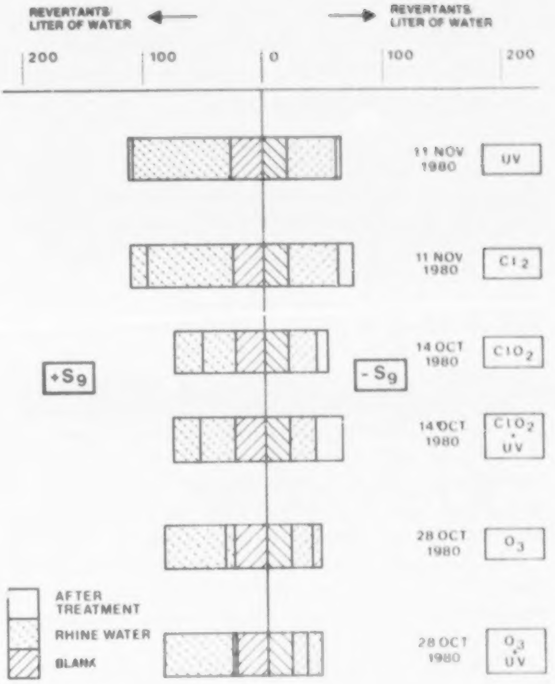

FIGURE 6. Changes in mutagenicity of stored Rhine water after disinfection by chlorine, chlorine dioxide, ozone, ultraviolet irradiation and some combinations (XAD-DMSO, *S. typhimurium* TA 98).

Table 5. Substances reduced in concentration by treatment of stored Rhine water.

Reten on inde	Compound	Concentration, µg/l. Raw water	After UV	After Cl_2	After O_3
109	Unknown aldehyde/ketone	0.3	0.3	—	—
118	Phosphoric thioic acid O,O,O-trimethyl ester	0.3	0.3	—	—
123	Unknown aldehyde/ketone	0.3	0.3	—	—
125	C_3-Benzene	0.1	0.1	—	—
212	1,3,3-Trimethyloxindole	0.3	0.3	0.3	—
215	Dicyclopentadiene	1	1	—	—
218	Dimethylquinoline	3	3	3	—
219	Dihydrotrimethylquinoline	10	3	—	—
224	2,6-Bis(*tert*-butyl) 2,5-cyclohexadiene-1,4-dione	1	1	1	—
226	3,3-Dimethyl-1,5 bis(isobutyl)bicyclo(3,1,0)hexanone-2	30	10	30	—
243	2-(Methylthio)benzothiazole	1	1	—	—
248	Trimethylquinoline	10	3	10	—
265	Unknown alkylphenol	0.1	0.1	0.1	—
301	Unknown chlorinated compound	3	3	3	0.1

[a]Denotes less than 0.1 (µg/l.)

The uncertainty this problem places on the Ames test results discussed has yet to be resolved.

Final Considerations

A retrospective epidemiological study among 1/3 of the population of The Netherlands showed a statistical association between chlorination by-products in drinking water and cancer of the esophagus and the stomach for males. An association with bladder cancer, as reported in the U.S. (*24*), was not found. Further indications were obtained that carcinogenic effects attributed to chlorination of surface water may in fact be partly due to carcinogens already present in the raw water.

The data presented suggest that a complex mixture of mutagens/carcinogens is involved. This means that generalizations of specific test results should be regarded with a certain degree of skepticism. It is against this background that the following conclusions and recommendations must be understood.

The increase of direct acting mutagens after treatment of stored river water with chlorine dioxide is similar to the mutagenic effect demonstrated after chlorination.

Ozone was found in these tests with Rhine water to reduce mutagenic activity both with and without metabolic activation.

Ultraviolet irradiation alone does not considerably influence the mutagenicity of contaminated water but it seems to enhance the diverging effects on the mutagenicity of chlorine dioxide and ozone.

An increase of mutagenicity after disinfection of the water studied for the *Salmonella typhimurium* TA 98 strain is always more pronounced without metabolic activation than with metabolic activation. In the case of Rhine water, chemical oxidation generally resulted in a decrease of the mutagenic activity (TA 98) after metabolic activation. This effect was more pronounced for $O_3 + UV > O_3 > ClO_2 + UV > ClO_2 > Cl_2 > UV$.

In all experiments practically no mutagenic activity for the *Salmonella typhimurium* TA 100 strain was demonstrated.

Although substantial differences in mutagenicity due to alternate disinfection techniques were detected, the identity of the causative mutagens is yet unclear.

The procedure applied for concentration and determination of the mutagenicity of water contaminants mainly detects the less volatile compounds. Furthermore the adsorption behavior of the mutagens on XAD is not sufficiently known. Yet, from a practical point of view, it seems justifiable from the data presented, to conclude that research in this area should focus more on the less volatile disinfection by-products than on the widely studied trihalomethanes.

Finally, within the context of the present results it may be concluded that oxidation and disinfection at the treatment station can most safely be achieved by ozonation, possibly in combination with ultraviolet irradiation. However, this leaves in many cases the problem of maintaining sufficient disinfectant residual in the water during distribution. Where this is needed, effective precursor removal, e.g., by coagulation and carbon adsorption, and a subsequent minimal safety disinfection is recommended. The data obtained so far have not indicated a substantial advantage of chlorine dioxide over chlorine for final safety disinfection.

The authors wish to thank Messrs. A. den Boer, M. J. Hart, H. J. van Kranen, C. F. van Kreijl, H. A. M. de Kruijf and P. Marsman for their valuable contribution to this study and their critical remarks in the preparation of this paper.

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Environmental Health Perspectives
Vol. 46, pp. 207-214, 1982

Presence, Introduction and Removal of Mutagenic Activity During the Preparation of Drinking Water in the Netherlands

by H. J. Kool,* C. F. van Kreijl,* E. de Greef* and H. J. van Kranen*

A survey of the presence of mutagenic activity in drinking water of 18 cities in the Netherlands revealed that in drinking water of 13 cities mutagenic activity could be demonstrated. The activity was detected in the Ames test after concentrating the organic mutagens with a XAD-4/8 procedure. Dose-related responses were observed with concentrates corresponding to 0.5 to 3.0 liters of drinking water.

A study of the changes in mutagenic activity during the preparation of drinking water in a few waterworks showed that breakpoint chlorination, transport chlorination and post chlorination increased the mutagenic activity, while ozonation only reduced the activity with metabolic activation. When adsorption on activated carbon powder was used, a certain reduction of mutagenic activity was observed. The use of activated carbon filters, however, removed the activity completely. The majority of organic mutagens present in drinking water concentrates were shown to be nonvolatile and relatively stable and probably consist of compounds with a molecular weight in the order of 200. These mutagens are not identical to the organics identified up till now in drinking water by standard gas chromatography/mass spectrometry analysis. Finally, a group of organic mutagens, which adsorbs only at pH 2–3 on XAD-4/8 (acid fraction), could be demonstrated in Ames-positive drinking waters.

Introduction

In the Netherlands, groundwater, surface water, and a mixture of both are used as raw water sources for drinking water. In particular, the rivers Rhine and Meuse serve as important sources for the drinking water supply; the two rivers provide the potable water for about 5 million people in the Netherlands (*1*).

In the Rhine and Meuse and in drinking water prepared from these rivers, several recognized mutagenic and carcinogenic organic compounds have been identified by Leer et al. (*2*), Morra et al. (*3*), and Zoeteman (*4*). Using the Ames Salmonella/microsome assay in combination with the XAD concentration procedure for organics, mutagenic activity in 50 ml Rhine and 500 ml Meuse water was demonstrated by Van Kreijl et al. (*5*) and Kool et al. (*6*).

By using the same procedure in a limited drinking water survey in six cities, mutagenic activity was shown in 0.5 to 1 liter drinking water in four of these cities by Kool et al. (*7*). A more extended survey of the mutagenic activity in drinking water of 18 cities in the Netherlands was subsequently carried out in a collaborative program with the Dutch Waterworks and the Netherlands Waterworks' Testing and Research Institute (KIWA Ltd.). The selection of the cities has been based on the raw water source, the storage facility and the different treatment processes applied in the preparation of drinking water.

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In this paper the preliminary results of this survey are presented. In addition, results of a limited study are shown of the changes in mutagenic activity (introduction and removal) during the preparation of drinking water. Finally some characteristics of the organic mutagenic fraction present in drinking water are briefly discussed.

Materials and Methods

XAD Resins

Amberlite XAD-4 and 8 were obtained from Serva GmbH, Heidelberg F.R., Germany. Purification by repeated Soxhlet extraction, control by GC analysis and storage of the resins in methanol were described previously by Kool et al. (*6*).

XAD Concentration Procedure

Drinking water samples were taken just before the water left the treatment plant. The water was filtered (under nitrogen pressure) through a 0.45 μm membrane filter before concentration. For about a 7 × 10^3-fold concentration, 160 liters of the filtered water were passed over columns containing 20 cm^3 XAD-4/8 at a flow rate of 4 bed volumes/min and at a constant temperature of 15°C. Elution of the adsorbed organic constituents took place with an appropriate volume of DMSO or acetone (≥ 1 bed volume). For lower or higher concentration factors, corresponding smaller or larger volumes of water were passed through the XAD column until the desired water/eluate ratio (*v/v*) was obtained.

Thin Layer Chromatography (TLC)

For the TLC fractionation of acetone concentrates of drinking water, preparative plates precoated with 2.0 mm of Silicagel-G (PSG Merck, Fertigplatten) were used. A volume of 2.0 ml acetone concentrate was applied to the plate with an automatic spraying device in the form of a small band (4.0 × 0.3 cm). The chromatograms were developed in one direction using ethyl acetate:isooctane (1:1) as the first solvent system. The plates were air-dried overnight and a second solvent system, benzene:methanol (4:1), was used. The plates were air-dried again prior to further investigation.

The developed plates were examined under ultraviolet light (366 nm) in order to mark the separate bands. The marked fractions (six) were collected by scraping off and collecting the adsorbents with a Pasteur pipet connected to a vacuum pump. The outlet of the pipet was fitted with a plug of glass wool. The organic material was recovered by eluting the pipet with 5 ml DMSO. The eluates were stored at -20°C prior to mutagenicity testing.

Gel Filtration on Sephadex LH20

The glass column (height 40 cm, 1 cm) was packed with Sephadex LH20 in dioxane–water (7:3) as described by Concin et al. (*8*). About 1.0 ml of a DMSO/XAD concentrate of drinking water was layered on the column, and subsequent gel filtration was performed with dioxane-water (7:3) as solvent. Fractions of 1 ml were collected with an automatic fraction collector. After measuring the absorbance at 263 nm, the fractions were pooled, diluted fivefold in water, reconcentrated on XAD-4/8 (bed volume 4 ml), and eluted with 5 ml DMSO. The concentrate was stored at -20°C prior to mutagenicity testing. Calibration of the column was performed by using two colored markers: Vitamin B_{12} (molecular weight 1355) and nitrofurazon (molecular weight 198).

Bacterial Strains

The *Salmonella typhimurium* strains TA 98 and TA 100 as described by Ames et al. (*9*) were used. They were stored frozen at -80°C in nutrient broth containing 10% DMSO.

Ames Salmonella/Microsome Assay

Mutagenicity testing of the organic concentrates of drinking water was carried out according to the plate incorporation assay described by Ames et al. (*9*).

The induction of microsomal enzymes with Aroclor 1254 and the preparation of the rat liver homogenates (S-9) has also been described by Ames et al. (*9*). In the S-9 mix, 0.075 ml of liver homogenate was added per milliliter of mix. All water concentrates were tested in three to five replicates, and the results were considered significant when a twofold increase above the background as well as dose response effects were observed. The deviation of the mean was usually below 20%. Routine controls to check for the presence of factors affecting bacterial growth were incorporated as described by Kool et al. (*6*).

Table 1. Types of drinking water investigated

City number	Drinking water prepared from
1–5	Ground water
6	Dune-infiltrated Meuse water
7, 8a, 9	Dune-infiltrated Rhine water
10–15	Bank-infiltrated Rhine water
16	River Meuse water
8b, 17, 18	River Rhine water

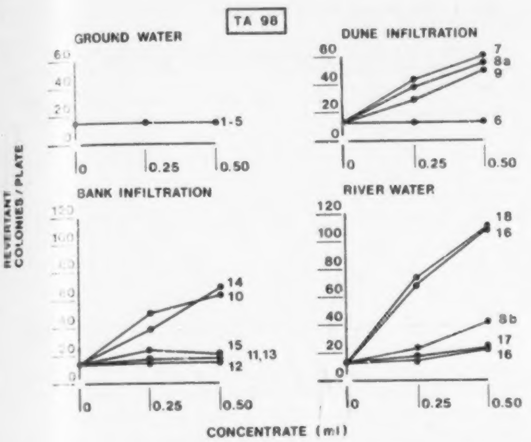

FIGURE 1. Mutagenic activity in drinking water of 18 cities in the Netherlands. The sampling, 7000–fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with *S. typhimurium* strains TA 98 and TA 100 are described in the text. The numbers refer to the 18 cities listed in Table 1, and each point represents the average of five plates.

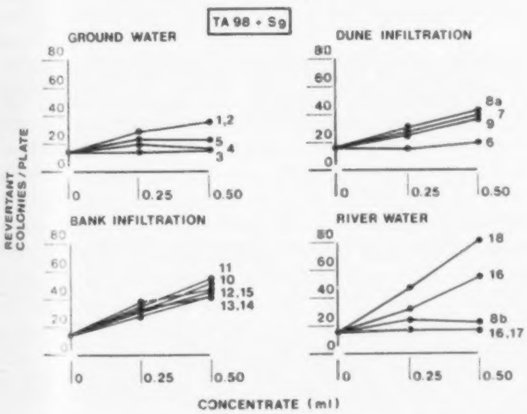

FIGURE 2. Mutagenic activity in drinking water of 18 cities in the Netherlands. Sampling, 7000–fold concentration with XAD-4/8, DMSO elution and mutagenicity testing as described in the text. The numbers refer to the 18 cities listed in Table 1, and each point represents the average of five plates.

Results

Mutagenic Activity of Drinking Water in 18 Cities

Our previous results, showing mutagenic activity in drinking water, all relate to drinking water prepared from the rivers Rhine and Meuse (7). To investigate whether these results are representative for other types of drinking water in the Netherlands, an extended survey in 18 cities (20 types of drinking water) was carried out. As shown in Table 1, these cities prepare their drinking water either from groundwater, surface water, or a mixture of both. In city 8 and city 16, drinking waters after two different treatment processes were investigated.

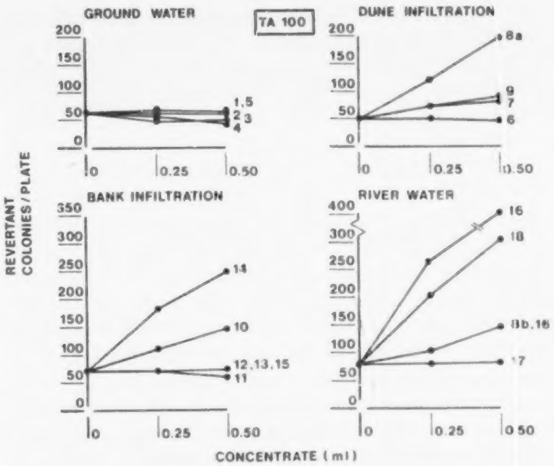

FIGURE 3. Mutagenic activity in drinking water of 18 cities in the Netherlands. Sampling, 7000–fold concentration with XAD-4/8, DMSO elution and mutagenicity testing as described in the text. The numbers refer to the 18 cities listed in Table 1, and each point represents the average of five plates.

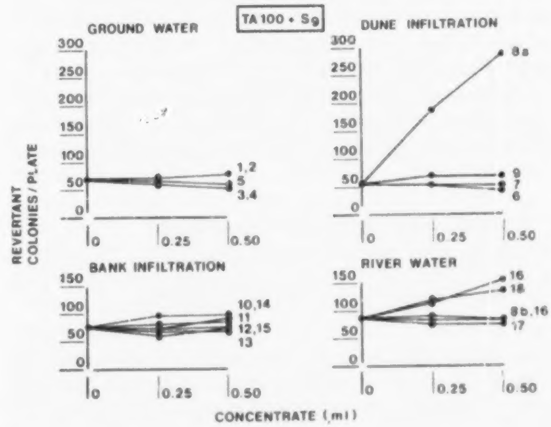

FIGURE 4. Mutagenic activity in drinking water of 18 cities in the Netherlands. Sampling, 7000–fold concentration with XAD-4/8, DMSO elution and mutagenicity testing as described in the text. The numbers refer to the 18 cities listed in Table 1, and each point represents the average of five plates.

The results of the survey are presented in Figures 1–4 and summarized in Table 2. When the

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Table 2. Summary of mutagenic activity present in 20 types of drinking water.

Raw water source	Type of storage facility	Number of drinking water investigated	Number of cities positive in the Ames test[a]			
			TA 98	TA 98 + S9	TA 100	TA 100 + S9
Ground water	—	5	0	2(1,2)	0	0
River/ground water	Dune infiltration	4	3(7, 8a, 9)	3(7, 8a, 9)	1(8a)	1(8a)
River/ground water	Bank infiltration	6	2(10, 14)	6(10-15)	1(14)	0
River water	Storage reservoir	5	3(8b, 16, 18)	2(16, 18)	2(16, 18)	2(16,

[a]Numbers in parentheses are city numbers.

cities are classified in four groups with regard to the raw water source and type of treatment, several interesting features are observed. Firstly, only two out of the five cities which prepare their drinking water solely from groundwater show low but significant mutagenic activity. Secondly, drinking waters (12 out of 15) in cities which prepare their drinking water from surface water or (in)filtrated surface water show mutagenic activity at least in one of the strains used.

Only one city in the categories "dune infiltration" (city 6) and "surface water" (one drinking water type of city 16) shows no mutagenic activity. Thirdly, the level and type of mutagenic activity observed seems to be more dependent on the type of treatment than of the type of raw water.

The results obtained showed to be very reproducible, since a second survey carried out six months later gave almost identical results (not shown).

Introduction and Removal of Mutagenic Activity During the Preparation of Drinking Water

The rivers Rhine and Meuse contain mutagenic activity as shown previously by Van Kreijl et al. (*5*) and Kool et al. (*6*). They demonstrated that this activity predominantly requires metabolic activation (S-9 mixture). In drinking water, however, the mutagenic activity is most pronounced without metabolic activation in many of the cities. This suggests that during drinking water preparation a change in mutagenic activity has occurred. Therefore, we studied the changes in the mutagenic activity in some of the waterworks. The effects of chlorine treatment (breakpoint chlorination, transport chlorination, post chlorination), ozonization, activated carbon adsorption and dune infiltration were examined. The results are shown in Figures 5–7.

Figure 5 (city 16) shows that the type of mutagenic activity of the raw water source is still present after storage for about 100 days in a reservoir. After breakpoint chlorination, however, the direct acting mutagenic activity in both strains was greatly increased. Activated carbon (powder) reduces the mutagenic activity in all cases but the reduction is less marked in strain TA 100. The slight increase of the direct acting mutagenic activity in both strains in finished drinking water may be due to post

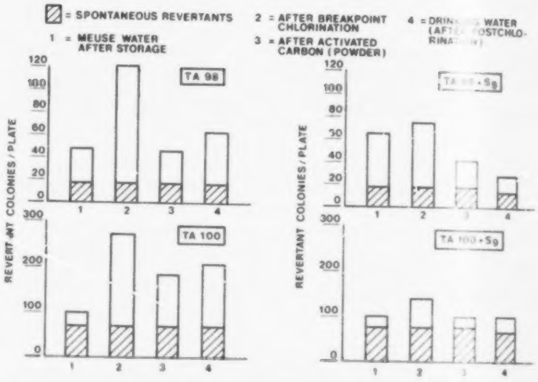


FIGURE 5. Course of mutagenic activity during the preparation of drinking water in city 16. The sampling, 7000–fold concentration of water samples, DMSO elution and Ames testing are as described in the text. The results correspond to about 1.5 liter of water per plate.

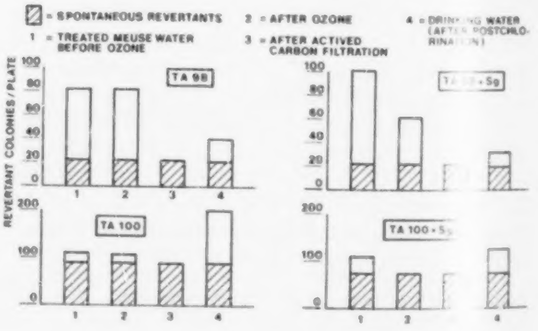


FIGURE 6. Course of mutagenic activity during the preparation of drinking water in city 16. The sampling, 7000–fold concentration of water samples, DMSO elution and Ames testing are as described in the text. The results correspond to about 3 liters of water per plate.

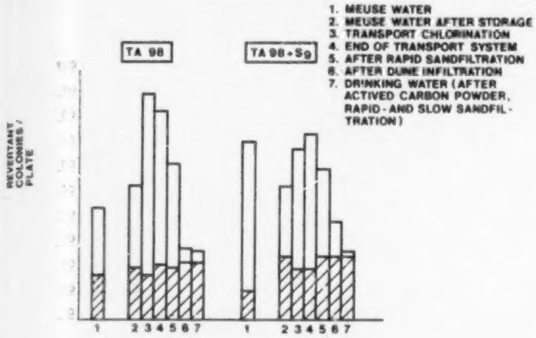


FIGURE 7. Course of mutagenic activity during the preparation of drinking water in city 6. The sampling, 8000–fold concentration of water samples, DMSO elution and Ames testing are as described in the text. The results correspond to about 2 liters of water per plate.

chlorination. Figure 6 (city 16) shows that an ozone treatment does not reduce the direct acting mutagenic activity with strain TA 98 and only partly reduces the activity with TA 98 + S-9. In this case the original mutagenic activity with TA 100 ± S-9 is so marginal, that no conclusion can be drawn with regard to the ozone treatment.

The mutagenic activity is completely removed in both strains by activated carbon filtration, while post chlorination introduces mutagenic activity in both strains again. Figure 7 (city 6) shows that transport chlorination also increases the mutagenic activity with strain TA 98 (± S-9). No activity was observed with strain TA 100. Rapid sand filtration hardly reduced the activity while dune infiltration reduced the mutagenic activity to a great extent. Finally the residual mutagenic activity is completely removed by adsorption on activated carbon (powder) in combination with a rapid and slow sand filtration step.

Characterization of the Mutagenic Fraction and Some Properties of the Responsible Organic Mutagens

By using the XAD-4/8 procedure for concentrating organic mutagens in drinking water, mutagenic activity can be detected in 13 out of 18 cities. Readsorption of the remaining compounds in the drinking water of city 18 (after passing the XAD column) on a second column at pH 2–3, however, revealed the presence of another class of organic mutagens (acid fraction) (*10*). Whether this class of organic mutagens generally is present in drinking water was investigated. Four drinking waters positive in the Ames test and one negative were studied. The four drinking waters with mutagenic activity also contained this class of organic mutagens which only adsorbs at pH 2–3, while the drinking water negative in the Ames test did not (not shown).

At present there is little information on the nature of the organic mutagens present in drinking water. Therefore, investigations are carried out to determine the chemical composition of the mutagenic fraction obtained by the XAD concentration procedure. We have reported previously (*10*) that the major part of the organic mutagens in city 18 are in the slightly polar nonvolatile fraction. They are still present after boiling the water and are not identical with the organic compounds already identified in drinking water by routine GC/MS analysis.

In an attempt to fractionate the organic mutagens, thin layer chromatography of acetone concentrates derived from stored surface water and drinking water prepared from this surface water has been carried out. The results in Figure 8 show that the organic mutagens active on strain TA 98 which are present in surface and drinking water predominantly are found in fraction 3. The results after TLC analysis suggest that the direct acting organic mutagens present in fraction 3 of the surface water concentrate are largely recovered in fraction 3 of the drinking water concentrate. The organic mutagens requiring metabolic activation in surface water, however, are more efficiently removed by the purification processes. The presence of organic mutagens which show an identical migration and fluorescence pattern on the thin layer plate suggests that the mutagenic compounds present in both fractions 3 may be the same. To estimate the molecular weight of the organic mutagens in this fraction, gelfiltration of Sephadex LH20 was carried out.

The results shown in Figure 9 indicate that the majority of the organic mutagens present in TLC fraction No. 3 has a molecular weight in the order of 200 assuming that these organics show the same behavior in Sephadex LH20, as the controls.

Discussion

In the Netherlands a limited survey by Kool et al. (*7*) on mutagenic activity in drinking water revealed that in four out of six cities mutagenic activity could be shown. All cities in this survey, however, prepare their drinking water from surface water.

To see whether these results are representative for drinking water in the Netherlands, a survey in 18 cities has been carried out including groundwater, surface water, and mixtures of both as raw water source. It was found that in 13 cities mutagenic activity could be demonstrated in 0.5 to 3 liters of drinking water (Figs. 1–4). The results of

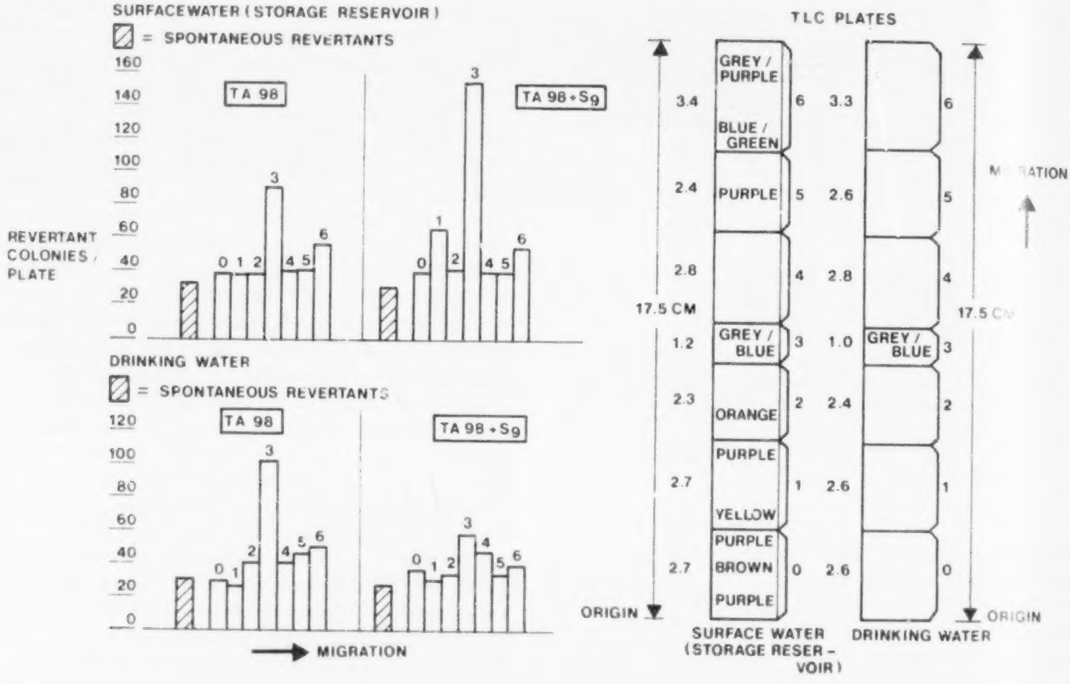


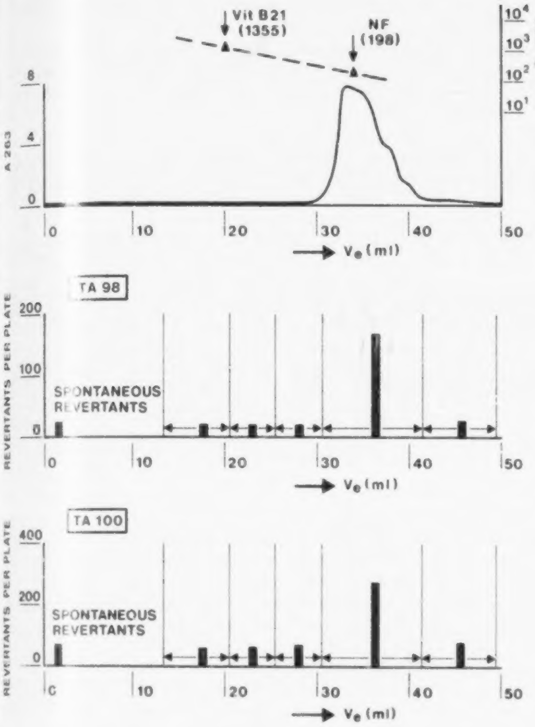
FIGURE 8. Fractionation of water concentrates prepared from storage reservoir water and drinking water by thin layer chromatography. The sampling, 9000–fold concentration of water samples from city 18, and elution with 60 ml acetone are as described in the text. The acetone concentrates were mixed with four volumes of H_2O and reconcentrated on a smaller XAD-4/8 column and again eluted with acetone (12 ml). These acetone concentrates (45,000–fold concentration) were tested by TLC. After incubation, six fractions were scraped off the TLC plate and eluted with 6 ml DMSO and assayed in the Ames test (TA 98 ± S-9).

this survey are not incidental because similar results in the 18 cities were obtained during a second survey six months later.

From these results it appeared that two out of the five cities which use groundwater as raw water source showed mutagenic activity, but this activity was only marginal (Fig. 2). Most cities (12 out of 15) which prepare their drinking water from surface water or infiltrated surface water clearly showed mutagenic activities. It is interesting that all cities which use bank-infiltrated river water as raw water source were positive in the Ames test with strain TA 98 + S-9 and that only one city in the dune infiltration category (city 6) and one (drinking water type of city 16) in the river water category did not show mutagenic activity in the Ames test. For further information about bank and dune infiltration see Piet and Zoeteman (*11*).

From these results the conclusion can be drawn that a proper combination of treatment processes may remove the organic mutagens to a high degree. The finding that mutagenic activity in Dutch surface water predominantly requires metabolic activation, while the activity in drinking water in many cities is most pronounced without metabolic activation (Figs. 1–4) indicates that a shift in the overall type of activity may have occurred due to different treatment processes. Cheh et al. (*12*) and de Greef et al. (*13*), who investigated whether the water quality will change after a chlorine treatment, reported an increase of mutagenic activity. Bull et al. (*14*) discussed the evidence for an increase of carcinogenic activity in chlorine treated water. To see how the changes in mutagenic activity take place in practice, several purification processes applied in different waterworks were investigated. In particular, a breakpoint chlorination dramatically increases the mutagenic activity (Fig. 5). However, transport chlorination (Fig. 7) as well as post chlorination (Fig. 6) do increase the mutagenic activity to a lesser extent. Ozonization of the water decreased the indirect mutagenic activity on strain TA 98, while the activity remained unchanged without metabolic activation (Fig. 6).

Considering the treatment with activated carbon powder, it is obvious that the removal of mutagenic



Fi RE 9. Gel filtration of a drinking water concentrate city 18) with Sephadex LH20. Sampling, 250,000-fold oncentration of drinking water on XAD-4/8, elution with)MSO and subsequent gel filtration were as described in he text. After measuring the absorbance at 263 nm, the ractions were pooled and, after dilution in water, econcentrated on XAD-4/8, eluted with 5 ml DMSO and ssayed for mutagenic activity with *S. typhimurium* trains TA 98 and TA 100.

activit is not very efficient (Fig. 5). Activated carbor filters, however, were shown to be very efficie in the removal of organic mutagens, because no mu agenic activity could be detected in the water vith the Ames test after this step. Other activa d carbon filtration experiments with six differe t activated carbon filters showed equivalent result (Kool, unpublished data).

Fin ly, dune infiltration in combination with activated arbon (powder) and slow sand filtration were shown o be very effective in a waterwork in removing or anic mutagens present in water (Fig. 7).

Up o now there has been little information on the na ure of the mutagenic compounds in drinking water Recently, however, Tabor and Loper (*15*) report d that mutagenic activity in a drinking water conce trate from Cincinnati may be due to the presen ce of isomeric chlorinated aliphatic ethers. Kool et al. (*7*) found that the mutagenic activity of a Dutch drinking water is observed mainly in the slightly polar nonvolatile fraction and that these compounds are not the same organics already identified in this type of drinking water by GC/MS.

These results indicate that these organic mutagens are not the same compounds as described by Tabor and Loper (*15*). Kool et al. (*10*) also observed another class of organic mutagen present in one type of drinking water. The presence of this class of mutagens (only adsorbed at pH 2–3 on XAD 4/8) was also detected in other Ames positive drinking waters. The behavior of this class of organic mutagens (acid fraction) during different water purification processes is under investigation.

Using thin layer chromatography we showed that the direct acting mutagenic fraction on TA 98 present in surface water is found at the same level in drinking water (Fig. 8). Furthermore, preliminary experiments using gel filtration on Sephadex LH20 with a DMSO concentrate of drinking water indicate that these organic mutagens probably have a molecular weight of the order of 200 (Fig. 9).

Finally the presence of mutagenic activity in the Ames test in drinking water of 13 cities raised the question whether these findings are significant for human health. To answer this question more research has to be carried out. Long-term animal studies with drinking water concentrates as well as additional *in vitro* biological assays and chemical analysis are presently carried out on the mutagenic fractions in order to obtain a better understanding of their health significance.

The authors wish to thank Mrs. M. Vredenbregt for the gift of the purified XAD resins and Prof. Dr. J. H. Koeman for critical reading of the manuscript. Also, the technical assistance of Mrs. M. de Vries, J. W. Louter, H. Nijholt and Mrs. M. Vredenbregt in a number of experiments is gratefully acknowledged. The research presented in this paper is supported in part by grant RID 80-1 from the Netherlands Cancer Society (Koningin Wilhelmina Fonds).

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Environmental Health Perspectives
Vol. 46, pp. 215-227, 1982

Use of Biological Assay Systems to Assess the Relative Carcinogenic Hazards of Disinfection By-Products

by R. J. Bull,* M. Robinson,* J. R. Meier*
and J. Stober*

Other workers have clearly shown that most, if not all, drinking water in the U.S. contains chemicals that possess mutagenic and/or carcinogenic activity by using bacterial and *in vitro* methods. In the present work, increased numbers of tumors were observed with samples of organic material isolated from 5 U.S. cities administered as tumor initiators in mouse skin initiation/promotion studies. Only in one case was the result significantly different from control.

In studies designed to test whether disinfection practice contributes significantly to the tumor initiating activity found in drinking water mixed results have been obtained. In one experiment, water disinfected by chlorination, ozonation or combined chlorine resulted in a significantly greater number of papillomas when compared to nondisinfected water. In two subsequent experiments, where water was obtained from the Ohio River at different times of the year, no evidence of increased initiating activity was observed with any disinfectant. Analysis of water obtained at the comparable times of the year for total organic halogen, and trihalomethane formation revealed a substantial variation in the formation of these products.

Considering the problems such variability poses for estimating risks associated with disinfection by-products, a model system which makes use of commercially obtained humic acid as a substrate for chlorination was investigated using the Ames test. Humic and fulvic acids obtained from two surface waters as well as the commercially obtained humic acid were without activity in TA 1535, TA 1537, TA 1538, TA 98 or TA 100 strains of *S. typhimurium*. Following treatment with a 0.8 molar ratio of chlorine (based on carbon) significant mutagenic activity was observed with all humic and fulvic acid samples. Comparisons of the specific mutagenic activity of the chlorinated products suggests that the commercial material might provide a useful model for studying health hazards associated with disinfection reactions by-products.

Introduction

Development and widespread application of coupled gas chromatography and mass spectrometry has provided a much greater appreciation of the number and variety of organic chemicals that can be found in most drinking waters (*1*). For the most part, the large numbers of chemicals which are identifiable are present at very low concentrations, usually in fractions of a microgram per liter (*1*). However, in total, the amounts of organic material found in drinking waters derived from surface sources are often in the range of several milligrams per liter, hardly a negligible amount. It is now recognized that a very small fraction of the chemicals present in drinking water lend themselves readily to analysis even with today's modern analytical tools.

As a consequence of the above considerations, efforts were begun to obtain an estimate for the general magnitude of the problem without expending the huge sums of money to test individual chemicals as they were identified. A number of research groups around the world began to examine test mixtures of organic chemicals extracted from drinking waters. The test systems employed varied from the application of the *Salmonella*/microsome assay (*2–4*) for mutagenic chemicals

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developed by Ames et al. (*5*) to chronic studies conducted in rodents (*6–8*). In each of these cases, carcinogenic and/or mutagenic activity was detected in finished drinking water. In the U.S., Loper and co-workers (*2*) reported mutagenic activity of organic chemicals concentrated from the drinking water of first five, then six U.S. cities by a technique developed by Kopfler, et al. of our laboratory (*9*). Following up on these initial observations it was shown that these concentrates also contained chemicals capable of transforming BALB/3T3 cells (*10*) and a preliminary report demonstrated the ability of certain of the samples to initiate tumors in the mouse skin (*8*).

The work of Bellar and Lichtenberg (*11*) in the U.S. and Rook (*12*) in Europe changed the view of drinking water problems substantially. These workers documented that the organic chemicals most commonly found in drinking water and usually at the highest concentrations were produced by chlorination. These chemicals were, of course, the trihalomethanes. From this initial observation has come the gradual appreciation of a problem which is a direct result of currently accepted methods for producing a microbiologically safe drinking water. Before this, chemical hazards in drinking water had been viewed simply as one of industrial and/or agricultural contamination of the source water. Now any assessment of health hazards in drinking water must consider the qualitative changes in chemical composition that arise from water treatment—in particular disinfection.

Our laboratory has been concentrating on two problems. First, efforts have been directed towards determining if chemicals obtained from drinking water can be demonstrated to be carcinogenic *in vivo*. Second, an attempt is being made to develop a base of information which can be applied to a wide variety of water supplies. This latter effort requires that sufficient understanding of processes which contribute to the formation and occurrence of carcinogenic chemicals in drinking water be developed so that the risks involved can be minimized. It includes consideration of the carcinogenic activity of the products of four alternative disinfectants; chlorine, chlorine dioxide, ozone and chloramine.

With regard to disinfection by-products it has been established that a substantial fraction of the organic material found in surface water sources is of natural origin. A substantial portion of this natural background is composed of humic and fulvic material that has been shown to be a major precursor of trihalomethanes following their chlorination. Recovery of the humic material from water in quantity is difficult, but humic acid obtained from peat is commercially available. The question is whether differences in the chemical properties of humic material obtained from these sources result in the formation of clearly different toxic by-products. Therefore, we report here an initial attempt at determining whether humic acid provides a good model for the generation of biologically active by-products of disinfection for more comprehensive testing.

Methods

Sample Preparation

The five cities studied were selected as being representative of water supplies contaminated by various sources, or as having no known contamination by municipal, industrial or agricultural sources. Miami was chosen as representing ground water containing large quantities of natural organic chemicals; New Orleans, a surface water source impacted by industrial wastes; Ottumwa, a surface water contaminated by agricultural runoff; Philadelphia, a surface water impacted by municipal waste; and Seattle, a pristine surface water obtained from a protected watershed in the Cascade mountains. In all cases finished drinking water was used.

The concentrated organics were prepared for EPA by Gulf South Research Institute by the procedure described by Kopfler et al. (*9*). The samples are concentrated from multiple 200 liter quantities of tap water by reverse osmosis using a cellulose acetate membrane (CA) with sufficient concentrated HCl added to maintain a pH of 5.5. The reject from the CA membrane is passed through a heat exchanger to maintain the water temperature at 15°C. Part of the reject stream is diverted through a Donnan softening unit to avoid salt precipitation. The CA permeate is adjusted to pH 10 and then concentrated by reverse osmosis using a nylon membrane. The nylon permeate is discarded. Both the CA and the nylon concentrates are then adjusted to neutral pH and extracted with pentane and methylene chloride. The aqueous phases are adjusted to a pH of 2 with HCl and again extracted with methylene chloride. For purposes of this study these fractions were combined and the solvent removed to produce the reverse osmosis extract sample (ROE).

The residual aqueous concentrate remaining after extraction with the organic solvents is passed through a column of purified XAD-2 resin at pH 2. Following the removal of metallic oxides and other inorganic agents by eluting with 1*M* HCl and distilled water the organics are then eluted from the column with 95% ethanol. The ethanol is removed from the eluate by vacuum distillation and eluates from both columns are combined to produce the XAD sample.

A series of six experiments has been initiated to examine the effects of seasonal variation on the ability of different disinfectant treatments to produce carcinogenic by-products. The testing of the first three of these has progressed to a point where results can be reported. Routine analysis for general water parameters, however, are only available for the last three experiments. Consequently no parallels between biological and chemical results are as yet possible. In the first three studies, settled, coagulated and filtered Ohio river water was subjected to chlorine, chloramine, chlorine dioxide, or ozone disinfection. Disinfectant demands were determined on all samples prior to adding the disinfecting agents. The disinfectant contact times varied but were designed to result in residuals that were at a practical minimum. The doses of disinfectants amounted to 2.0-2.5 mg/l. chlorine, 1.0-3.0 mg/l. ozone, 2.0-3.0 mg/l. chlorine dioxide, and 2.0-3.0 mg/l. combined chlorine. A control consisted of filtered, but nondisinfected water. Waters and concentrates obtained in March, July and October of 1980 were assayed for various physical, chemical and microbiological parameters for quality control purposes (data not shown). All water samples were then concentrated by reverse osmosis using a cellulose acetate membrane. The May 1977 samples were concentrated 100- to 180-fold. Subsequent samples have all been concentrated to 400-fold, using freeze-drying to supplement reverse osmosis concentration where necessary.

Humic and fulvic acids were isolated from two natural lakes, Black Lake and Lake Drummond, by Christman and co-workers of the University of North Carolina as they have described previously (*13*). The major differences between these two lakes is the distribution of the total organic carbon between the recoverable humic and fulvic acid fractions. Black Lake contains a much higher proportion of its TOC as humic acid. Both lakes are remote from direct industrial contamination. Commercial humic acid was obtained from Fluka of Switzerland. These materials were prepared by adding 4 g of humic material to 500 ml deionized water. The pH was adjusted to 12 with NaOH and the solution was stirred overnight in the dark. The solution was then centrifuged at 16,000*g* for 20 min and the supernatant was brought to pH 3 by addition of HCl. The centrifugation was repeated. The supernatant was diluted to 1 liter, and the pH was adjusted to 7. The resulting total organic carbon (TOC) content was 2.5 g/l. Chlorination of the humic solution was accomplished by adding enough NaOCl (pH 12, 10 g/l.) to give a chlorine to carbon ratio of 0.8:1. After readjusting the pH to 6, the solution was diluted with sufficient deionized water to yield a TOC concentration of approximately 1 g/l. The solution was kept in a Teflon-capped bottle with no head space at room temperature for 5 days, at which time chlorine consumption was essentially complete. The final pH of this solution was 2.5 and the total chlorine residual was less than 1 ppm. The sample was then maintained at 4°C until time of assay for mutagenic activity. At the time of testing in Salmonella strains, the pH was adjusted to 7.

Analytical Methods

Measurements of trihalomethane (THM) concentrations, total organic halogen (TOX) and total organic carbon (TOC) were performed by Alan Stevens and his staff of the Drinking Water Research Division of the Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati or by Dr. Robert Lingg of the Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati. Trihalomethane concentrations were determined by using the purge and trap method described by Bellar and Lichtenberg (*14*). Total organic halogen (TOX) was analyzed directly in ambient water samples by using a Dohrmann Total Organic Halogen Analyzer. In studies involving chlorination of humic and fulvic acids 0.2 ml of the sample was diluted to 50 ml before the analysis was performed. Total organic carbon (TOC) analyses were performed using the Dohrmann Total Organic Carbon Analyzer. Again ambient water samples were analyzed directly and chlorinated humic acid samples were diluted from 1.0 ml to 50 ml before analysis.

Mouse Skin Initiation/Promotion Assays

SENCAR mice were obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. The SENCAR mice are a strain of mice selected for their sensitivity to developing skin tumors in response to 7,12-dimethylbenz(a)anthracene (DMBA) (*15,16*). The animals were 6 to 9 weeks of age when started on study. The dorsal hair of each mouse was shaved with electric clippers three days before the initial dose of the test compound and then shaved once weekly during application of the promoting agent to facilitate the application and the observation of tumor occurrence. All animals received distilled water and Purina Laboratory Chow *ad libitum* throughout the study.

Experiments with the five cities concentrates utilized total doses of 150 mg/kg (4.5 mg/mouse) of combined ROE fractions and the XAD samples applied subcutaneously in the back of the mice in six injections over a 2 week period. The vehicle used was 0.1 ml Emulphor (a polyoxyethylated vegetable oil). Two weeks after the last injection, a promotion schedule was begun involving the application to the shaved back of each mouse of 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) 3 times weekly in 0.1 ml acetone. After 20 weeks of application, TPA treatment was suspended and the animals were held for observation for an additional 28 weeks.

During the course of this and the following experiments the animals were weighed weekly and observed for tumor incidence. The incidence of both papillomas and carcinomas was charted weekly. Skin lesions were included in the cumulative papilloma count when they persisted for a minimum of 3 weeks. Following completion of the promotion period the animals are held for observation until they reach one year of age and then are sacrificed. Major organs and all grossly observed lesions are sectioned and fixed in a 10% buffered formaldehyde solution for subsequent histopathological diagnosis (histopathological confirmation made by Dr. John Orthoefer, HERL/Cincinnati).

In each of the three studies involving aqueous concentrates doses were administered subcutaneously in the shaved dorsal area of the mice. A total dose of 1.5 ml of the aqueous concentrates (6 × 0.25 ml) was given per mouse over a 2 week period. Negative controls received the same volumes of normal saline. As positive control either DMBA in doses of 7.5 or 25 μg/mouse or urethane at a dose of 9 mg/mouse was administered by the same routes. Both compounds were given subcutaneously in a 10% Emulphor vehicle. Two weeks following the last initiating dose, a tumor promotion schedule was begun wherein the mice received TPA topically to the shaved dorsum three times a week for 20 weeks, at a dose of 1.0 μg TPA in 0.2 ml acetone per application. It should be noted that in the first experiment (May 1977) a slightly different promotion protocol (than other experiments) was used. This involved application of 2.5 μg TPA/mouse three times weekly for 18 weeks. Due to the high sensitivity of the SENCAR mouse to the irritant effects of TPA at this dose, the dose was reduced in all subsequent experiments. For each group treated with TPA there was a corresponding group of 20 or 25 animals receiving acetone with no added TPA.

Assays for Mutagenic Activity

Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 were generously provided by Dr. Bruce Ames (University of California, Berkeley). Liver homogenate fractions (S9) were prepared from male Sprague-Dawley rats which had previously received a single IP injection of a PCB mixture (Aroclor 1254, Analabs, New Haven, Conn.) at a level of 500 mg/kg 4 days prior to sacrifice (*17*). Assay for mutagenic reversion of the test bacteria to histidine independence were performed according to the standard plate method of Ames (*5*). All assays were carried out in triplicate at three sample concentrations both in the absence and presence of S9 (25 μl/plate). Positive controls were assayed concomitantly with the test samples. For assays without S9 activation, these consisted of sodium azide for TA 1535 and TA 100; 2-nitrofluorene for TA 1538 and TA 98; and 9-aminoacridine for TA 1537. For assays with S9 activation, 2-aminoanthracene was used for all strains. Specific genetic markers (histidine dependence, ampicillin resistance, UV sensitivity and crystal violet sensitivity) were confirmed for each strain before use in assays.

Results

Five Cities Samples

The results obtained with the combined reverse osmosis extracts (ROE) from the five cities are shown in Figure 1. Although in all cases the tumor response was higher than in the control group, the sample obtained from Ottumwa stands out. It is notable that the increase in the development of tumors occurred after termination of the promotion schedule.

Results obtained with XAD fractions followed a similar course (Fig. 2). Here all samples again ended up at a higher tumor incidence than that observed in the control group. An excess in the total number of observed tumors per mouse can be used as evidence of carcinogenicity. No significant differences among proportions of animals with and without tumors for the ten test groups and control were found. This negative result might be due in part to the relatively low response rate (10–30%, $N = 40$ in each group). This type of data is called censured data, since both premature deaths and the end of the study precludes the full expression of the measured parameter in this case tumor incidence. Graphical comparisons did not reveal any gross differences in censoring patterns among the groups. An additional type of evidence for carcinogenicity would be a shorter time to first tumor distribution in exposed mice compared to control mice. Since the longest response times are observations that are censored the estimates of survival time (time without tumor) are truncated at the end of the study period. As a result, the mean time to first tumor will be underestimated, since very long times are not observed. However, if tumors developing very late in the study period may be due to other factors than exposure (e.g., age), then the time to tumor related to exposure may not be underestimated.

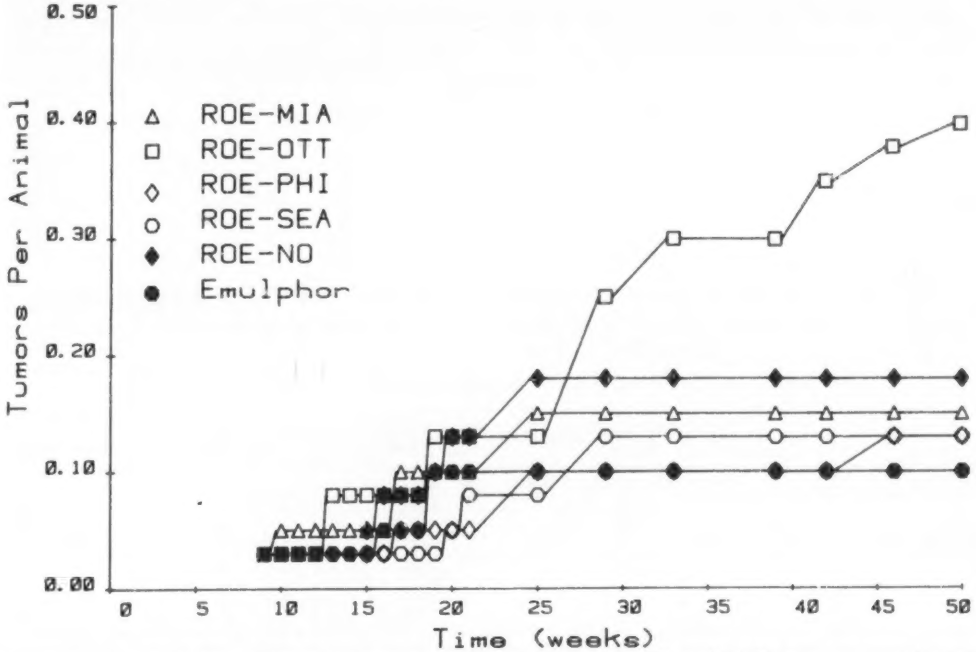

FIGURE 1. Tumor incidence through 50 weeks after an initiating dose of 150 mg/kg ROE (reverse osmosis extract) sample applied subcutaneously. Animals received 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a tumor promoter three times a week from weeks 0 to 20. Forty animals were included in each group. Emulphor was used as vehicle for ROE and the TPA was given in acetone.

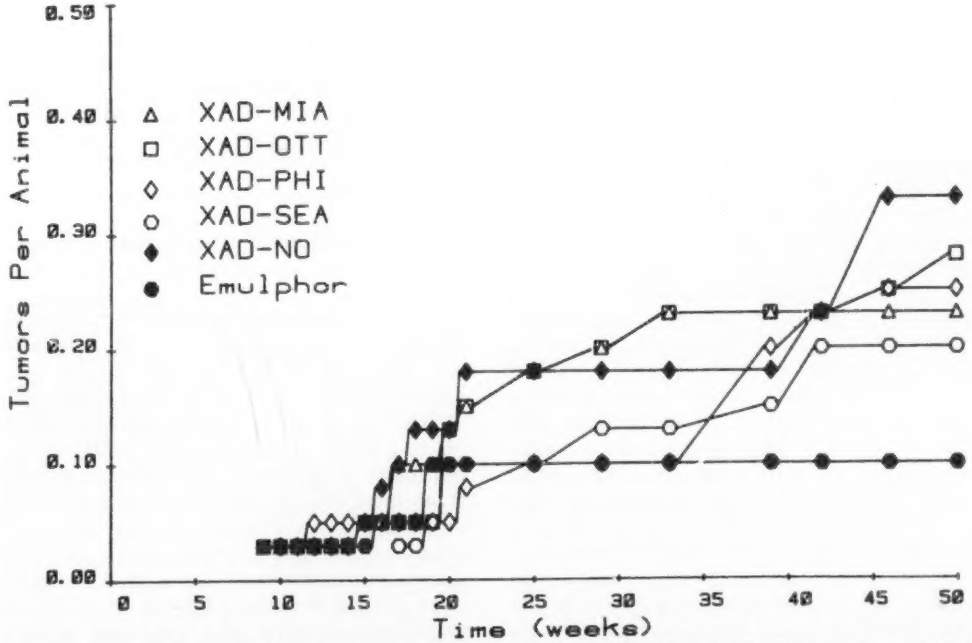

FIGURE 2. Tumor incidence through 50 weeks after an initiating dose of 150 mg/kg XAD (ethanol extract of XAD-2 resin containing residual ROE concentrate) sample applied subcutaneously. Animals received 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a tumor promoter three times a week from weeks 0 to 20. Forty animals were included in each group. Emulphor was used as vehicle for XAD and the TPA was given in acetone.

Table 1. Comparisons of mean time to first tumors distribution among the control and five cities samples

City	Mean time to first tumor, weeks		
	ROE[a]	XAD[b]	Comb
Ottumwa	44.2	44.3	44.
Miami	45.0	46.2	45.
Seattle	46.1	48.6	47.
Philadelphia	45.9	48.5	47.
New Orleans	45.1	47.6	46.
Vehicle control[c]	–	–	48.

[a] ROE = combined reverse osmosis extract.

[b] XAD = residual aqueous concentrate from ROE is collected on XAD-2 resin column and then eluted with ethanol.

[c] Vehicle was 10% Emulphor (a polyoxyethylated vegetable oil applied in six injections of 0.25 ml each).

Table 2. Skin tumor initiation by reaction products of alternate disinfectants; study 1.[a]

Sample[b]	Concentration factor[b]	No. animals with tumors	Total tumors	Tumors/animal × 100
Nondisinfected	102	0/25	0	0
Chlorine	106	4/25	5	20
Chloramine	142	5/25	8	32
Chlorine dioxide	168	0/25	0	0
Ozone	186	7/25	9	36
Saline	—	1/25	1	4
DMBA	—	16/25	35	140

[a] Substrate was settled, coagulated, and filtered Ohio River water. Total dose 1.5 ml (6 × 0.25 ml) subcutaneously at the concentration factor indicated. Animals were subjected to a promotion schedule involving application of TPA at a dose of 2.5 μg/mouse, 3 times per week for 18 weeks.

[b] After treatment indicated water was subjected to reverse osmosis with cellulose acetate to the level indicated by the concentration factor (initial volume/final volume).

The mean time to first tumor for each animal was used as the response variable in the following analysis. Comparisons of the distribution of these values for the XAD and ROE concentrates were made within each of the five city groups, using both the generalized Savage and the generalized Wilcoxon Statistic. Since no significant differences were found, the XAD and ROE groups were combined for each city to provide larger groups for the comparison of cities and control. No significant differences were found among the time to first tumor distributions of the five cities and the control using both test statistics ($p > 0.20$). However, all cities did have a shorter mean time to first tumor (Kaplan-Meier product limit estimates) than the control with Miami and Ottumwa being over two and three weeks shorter, respectively (Table 1). An alternative technique to analyze this type of data is the proportional hazards (or Cox regression) model. In this analysis, both concentrates from Ottumwa showed regression coefficients significantly different from zero, indicating a shorter mean time to first tumor in the Ottumwa group than in the controls. However, no other city concentrate group shows a significant difference from the control. It is notable, however, that all the ROE fractions resulted in shorter mean times to first tumor, ranging from a decrease of 1.9 weeks with the Seattle sample to a decrease of 3.8 weeks with Ottumwa. The XAD samples on the other hand, were much closer to the mean time to first tumor for the control animals with the exception of Ottumwa.

Alternate Disinfectant Reaction Products

The results of the first experiment testing aqueous concentrates from alternatively disinfected drinking waters are shown in Table 2. The overall chi-square analysis comparing the proportion of mice with tumors in the disinfectant groups (saline excluded) was highly significant ($p < 0.001$). It was found that most of the tumors (classified as papillomas by gross observation) were observed in groups of which 16, 20 and 28% of the animals treated were found to have tumors, respectively. When the overall chi-square was partitioned, these treatments combined were all significantly different from the combined responses observed with the nondisinfected water (0%) and chlorine dioxide treated water (0%). Although there were small differences in the degree to which these samples were concentrated by reverse

Table 3. Summary of macroscopically observed lesions at autopsy in alternate disinfectant study 1.[a]

	Skin tumors[b]		Systemic tumors[c]		
	Papillomas	Squamous carcinomas	Lung adenoma	Mammary tumors	Stomach tumors
Nondisinfected	0	1	0	0	0
Chlorine dioxide	0	0	4 (3)[d]	3	1
Chloramine	1	2	5 (4)[d]	0	0
Chlorine	1	3	2	0	0
Ozone	2	2	1	0	0
Saline	0	1	1	2	0
DMBA	8 (6)[d]	1	0	0	0

[a] Sample collected in May 1977.

[b] Skin tumors include only those animals promote with TPA (25 mice/group).

[c] Systemic tumors include both promoted (TPA) and nonpromoted animals (50 mice/group).

[d] Number of animals when not same as tumor count.

Table 4. Incidence of papillomas in alternate disinfectant studies.[a]

	Animals with papillomas, %					
Sample	Control[b]	Non-disinfected	Ozone	Chlorine	Chlorine dioxide	Chloramine
May 77	4	0	28	16	0	20
Nov 78	15	20	13	10	18	23
Mar 80	13	20	8	18	15	15
Average	11[c]	13	16	15	11	19

[a] Results in SENCAR mice after 1 year on study (40 mice/exposure group).

[b] Control animals injected with 0.9% saline.

[c] Historical control incidence is 8 ± 3% based upon six experiments with a total of 190 animals.

osmosis, there was no correlation between tumor response and degree of concentration ($r = 0.4$, $p \leq 0.50$). Consequently, we concluded that the differences were probably due to the reactions between disinfectants and trace organic material present in the water.

Papillomas are nonmalignant tumors and in themselves are not life-threatening. Burns and Albert (*18*) have shown that although carcinomas can arise from or independently of papillomas, the number of papillomas which arise are predictive of later carcinoma development. In Table 3 it can be seen that greater development of carcinomas did occur generally in the groups having more papillomas even though the incidences do not parallel precisely. Most of the carcinomas are to be found in the chloramine, chlorine and ozone-treated water groups. However, the response level of malignant tumors was too low to be statistically significant ($p = 0.36$).

When the numbers of systemic tumors that were observed on termination of this study were examined (Table 3) it was found that eight systemic tumors were found in the chlorine dioxide group. This included four lung adenomas (in three animals), three mammary gland tumors and one stomach tumor in the 50 animals treated with this sample (this includes 25 animals which did not receive topical TPA applications). Increased numbers of lung adenomas (5) were also observed with animals that received the chloramine-treated water. These tumors were not observed at all with the nondisinfected water. Only one lung adenoma and two mammary tumors were observed with the 50 animals injected with saline. The overall chi-square analysis comparing the number of animals with systemic tumors for this experiment is significant at $p = 0.05$ and the chlorine dioxide group is significantly different from the combined nondisinfected and saline controls at $p = 0.01$. Other treatments were not significant different statistically.

It was obvious that a single experiment of this type provides far too little information upon which to generalize concerning the relative safety of alternate disinfectant reaction products. Consequently, a series of five additional experiments has been initiated to examine the repeatability of the first experiment over the course of several seasons. Two of these five experiments have progressed to the point where the development of skin papillomas can be analyzed. Table 4 summarizes the results obtained from the experiment just described and these two

Table 5. Seasonal variations in the production of total organic halogen (TOX) and trihalomethane (THM) with alternate disinfectants using Ohio River water.

	Sampling date		
	3/80	8/80	11/80
TOC, mg/l.			
Nondisinfected	1.7	2.7	2.9
Ozone	1.7	2.4	3.2
Chlorine	1.8	2.4	2.4
Chlorine dioxide	1.7	2.6	2.5
Chloramine	2.0	2.7	3.1
TOX, mg/l.			
Nondisinfected	0.013	0.021	0.020
Ozone	0.011	0.023	0.041
Chlorine	0.053	0.237	0.169
Chlorine dioxide	0.017	0.060	0.042
Chloramine	0.026	0.064	0.065
THM, mg/l.			
Nondisinfected	0.0006	N.D.	0.0001
Ozone	N.D.	N.D.	0.0001
Chlorine	0.0083	0.080	0.065
Chlorine dioxide	N.D.	N.D.	0.0001
Chloramine	N.D.	0.0001	N.D.

subsequent experiments. It is clear that these latter two experiments are providing a different picture than that seen in the first experiment. A major difference is that a response rate greater than that observed in the control group is being observed in both experiments for the nondisinfected water. This might be the result of the greater concentration factor involved in these experiments compared to the first. All samples were uniformly concentrated 400-fold by a process initially involving reverse osmosis, but followed by freeze-drying. Whatever the reason, it is clear that the carcinogenic activity associated with alternatively disinfected samples did not significantly exceed that in the nondisinfected samples in these two experiments.

It should be pointed out that if taken in total and against historical controls, the average tumor incidence in the chloramine group is almost double the simultaneous control incidence and more than twice the historical control response. In addition, this increased incidence has been consistent across all three experiments. Although the differences are not statistically significant when compared to the simultaneous controls, if this trend is maintained through the remaining three experiments statistical significance may well be achieved.

There has been insufficient time since the completion of the latter two studies to confirm the gross pathological findings at terminal sacrifice. Consequently, comparison of the systemic tumors between experimental groups is not yet possible.

Data obtained from analyses of the nonconcentrated water used in subsequent experiments provides some insight into the variability of these results (Table 5). The organic chemical content of the Ohio River tends to be at a minimum during late winter and early spring and increases 60-80% with the lower flow of later summer and fall. These changes result in even more dramatic effects upon disinfection by-products. For example, total organic halogen (TOX) concentration is increased much more dramatically by chlorine, chlorine dioxide and combined chlorine in late summer than in the sample collected in March. In the case of chlorination, the trihalomethane (THM) portion of the TOX is 10-fold that observed in March. It should also be noted that under the conditions of late fall, ozone is capable of significantly increasing the TOX, whereas no significant alterations are observed in March or August.

Mutagenesis Assays

No net increase in the number of revertants was observed with *Salmonella* strains TA 1535, TA 1537 or TA 1538 treated with commercially available humic acid either before or after the addition of chlorine. As shown in Figure 3 the sample of humic material prior to the addition of chlorine was also without activity in strains TA 98 and TA 100 in the presence or absence of rat liver S9 fraction. However, if the sample is treated with chlorine it gives a clear cut response with both TA 98 and TA 100. A linear dose-response curve was observed in both strains. This activity is apparently destroyed by the incorporation of Aroclor 1254-induced rat liver S9 fraction.

In Table 6 the activity of the humic and fulvic acid fractions from two natural lakes (Lake Drum-

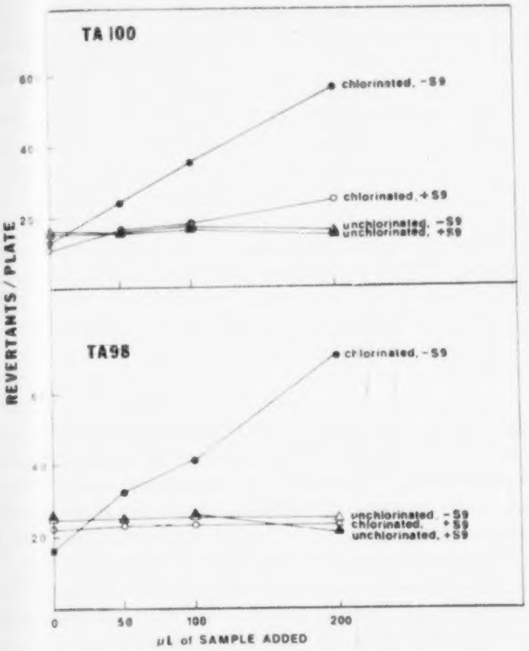

FIGURE 3. Effect of chlorination on the production of mutagenic activity in soiutions prepared with commercially available humic acid. The Ames Salmonella/microsome assay and chlorination of samples were performed as described in Methods. Assays were carried out both with and without S-9 added. Each point represents the average of duplicate determinations.

mond and Black Lake) along with that obtained using humic acid obtained commercially from Fluka are shown. In all cases involving the humic acid fraction, the same clear dose-response relationship is seen with the chlorinated material. The unreacted material was without activity as indicated earlier for the commercial humic acid. In all cases the activity of the chlorinated material was decreased by the incorporation of rat liver S9 fraction. It is notable in Table 7 that the degree of chlorine incorporation appears to be quite similar. The TOX/TOC ratio was 0.34 with Black Lake humic, 0.34 with Lake Drummond fulvic, 0.36 with Black Lake fulvic, 0.38 with Fluka humic and 0.44 with Lake Drummond humic. It appears that the specific mutagenic activity relative to either TOC or TOX is virtually identical for TA 100 with humic and fulvic acids from all three sources. In the case of TA 98, however, the specific activities observed ranged from no measurable mutagenic activity with the chlorinated Lake Drummond fulvic acid to a maximum specific activity with the chlorinated Lake Drummond humic acid. Chlorinated fulvic acid from Black Lake gave approximately 21 to 58% of the specific mutagenic activity in TA 98 when compared to the three sources of humic acid treated with chlorine.

Discussion

The present work adds to growing evidence that chemicals with carcinogenic activity are found in

Table 6. Comparison of mutagenic activities in chlorinated humic and fulvic solutions derived from a commercial source and two natural lakes.

Source	Volume added per plate, μl	Total revertants/plate[a] TA 98 −S9	Total revertants/plate[a] TA 98 +S9	Total revertants/plate[a] TA 100 −S9	Total revertants/plate[a] TA 100 +S9
	No addition (A)	15 (6)	25 (6)	152 (4)	127 (8)
	(B)	18 (2)	29 (2)	160 (18)	170 (27)
Fluka humic	50	32 (3)	23 (5)	237 (3)	161 (10)
	100	41 (13)	23 (1)	354 (9)	169 (28)
	200	70 (5)	23 (1)	559 (27)	264 (63)
Black Lake humic	50	45 (17)	32 (13)	299 (17)	203 (34)
	100	66 (11)	54 (3)	448 (20)	281 (49)
	200	110 (1)	73 (33)	653 (73)	343 (5)
Lake Drummond humic[b]	50	19 (10)	28 (10)	203 (15)	154 (11)
	100	31 (4)	33 (11)	233 (36)	152 (6)
	200	59 (9)	32 (20)	309 (20)	203 (0)
Black Lake fulvic	50	21 (1)	18 (8)	226 (42)	145 (18)
	100	21 (13)	26 (5)	375 (126)	281 (97)
	200	44 (1)	25 (13)	561 (16)	215 (42)
Lake Drummond fulvic	50	19 (9)	23 (3)	328 (5)	286 (24)
	100	20 (2)	22 (10)	395 (24)	303 (6)
	200	25 (7)	26 (9)	540 (63)	409 (11)

[a] Values are mean values of duplicate place counts with range values in parentheses. Humic and fulvic samples were assayed in separate experiments with control values indicated by (A) and (B), respectively.

[b] Significant precipitation of material occurred during acidification step in sample preparation. This resulted in a TOC concentration approximately 25% of other humic acid samples (see Table 7).

Table 7. Specific mutagenic activities of chlorinated humic and fulvic acids.

Source	TOC, mg/ml[a]	TOX, mg/ml[b]	Net revertants/mg TOC[c]		Net revertants/mg [illegible]	
			TA 98	TA 100	TA 98	TA [illegible]
Fluka humic	0.92	0.35	309	2323	811	[illegible]
Black Lake fulvic	1.01	0.34	450	2382	1335	[illegible]
Lake Drummond humic	0.69	0.25	181	3003	508	[illegible]
Lake Drummond humic	0.25	0.11	857	2694	1963	[illegible]
Lake Drummond fulvic	0.76	0.26	ND[d]	2367	ND	[illegible]

[a] Total organic content; analysis conducted by R. Lingg and R. Kaylor, HERL, USEPA, Cincinnati.
[b] Total organic halogen content; analysis provided by A. Stevens of MERL, USEPA, Cincinnati.
[c] Values are based on dose-response data from assays without S9 added.
[d] ND = no detectable mutagenic activity.

drinking water. Data showing an increased tumor incidence and a reduction of the mean time to first tumor in mouse skin initiation/promotion assays demonstrates the presence of tumor initiating activity in samples obtained from at least one city. Although not statistically significant, increases in tumor incidence and reduced mean times to tumor were observed in the other four cities studied. These data provide confirmation of the presence of chemicals with carcinogenic activity in the same samples which produced mutagenic affects in Salmonella strains TA 98 and TA 100 (*2*) and increased transformation in BALB/3T3 cells (*10*).

The importance of these results lies strictly in the fact that a tumorigenic effect can be seen in an intact animal model. These data should not be understood as demonstrating that cancer hazards are any greater in Ottumwa than the other cities studied. Nor are there implications of a specific level of risk attributable to cancer causing agents in any of these waters. At least two factors militate against the use of mouse skin initiation/promotion studies for assessing relative risks. First, the system responds selectively to different carcinogen classes, giving rise to a large number of false negative results (*19,20* and Bull et al., unpublished data). Secondly, the utilization of a promoting agent greatly enhances the tumorigenic effect of tumor initiators. In certain instances (e.g., topical ethyl carbamate application) it is virtually impossible to produce tumors in the absence of promotion.

Given the fact that a hazard of undefined proportions can be seen in drinking waters two very important questions must be addressed. The first of these is, "what is the source of this carcinogenic material?" The second is, "what is the magnitude of the risk to the consuming population?"

Several authors have shown that drinking water treatment including chlorination substantially increases the level of direct acting mutagens (*3,21–23*). Organic chemicals resulting from the treatment of wood pulp with chlorine (*24,25*) also result in production of direct acting mutagens in Salmonella tester strains. Ozonation of municipal wastewater has been shown to increase both the direct and indirect mutagenic activity present (*26*). Kool et al. (*22*) demonstrated that chlorine dioxide also resulted in increased levels of direct acting mutagens in strain TA 98. Consequently, it is clear that material present in drinking water sources is capable of reacting with disinfectants to produce biologically active products.

Testing of aqueous concentrates of alternately disinfected water as tumor initiators in the mouse skin illustrates many of the problems that arise in hazard evaluations of complex mixtures. Eventually such evaluations must deal with the individual processes that impact the chemical composition of the mixture to which man is exposed. In the first experiment a rather straightforward conclusion that reactions of ozone, chlorine, chloramine and chlorine dioxide resulted in a clear increase in the carcinogenic activity relative to that in the source water seemed possible. Subsequent experiments, however, failed to confirm this pattern. A major difference was that the nondisinfected water appears to possess activity not apparent in the earlier experiment. If one can assume that the series of analyses of water performed in 1980 reflects gross seasonal variations in the organic content and the reactivity of this material with disinfectants, it is clear that the variability suggested by the tumorigenesis data is clearly possible. Therefore, it becomes clear that consideration must be given to the variability in the source on the interpretation of results.

A second aspect of this variability is that disinfectants are very reactive chemically. Although the measures supplied in Table 4 concentrate on one aspect of disinfectant reactions, halogen substitution, all the alternate disinfectants are strong oxidants. Given the presence of biologically active chemicals in a source water it is not unlikely that such chemicals will be destroyed by disinfectants as easily as new chemicals with biological activity might be produced.

A third major source of variability is that testing is obviously being conducted at the limits of the sensitivity of this assay. This makes it predictably difficult to differentially determine the contribution of disinfectant to formation of carcinogenic products from the variations of the source contribution. Bioassays based on toxicological effects do not distinguish among the responsible chemical species. Therefore, it becomes very difficult to determine what strategy is necessary to reduce hazards or even to arrive at a circumstance which results in the lowest overall risk because of an inability to develop the data for systematic decision making.

Contributing to the lack of systematic or predictive information is the selective responsiveness of certain bioassays to certain classes of chemical carcinogens mentioned above. The mouse skin certainly suffers from this difficulty (*20*). Consequently,' future work must take an approach which comprehensively handles the question of relative carcinogenic hazards irrespective of the chemical classes present. An approach involving the use of a matrix of testing systems to define relative carcinogenic hazards has been previously described (*19*).

The above described difficulties argue for a better fundamental understanding of the chemical reactions that give rise to mutagenic, carcinogenic and otherwise toxic chemicals during disinfection of drinking water. It has been fairly well established that the organic substrates producing trihalomethanes and other reaction products of chlorination are quite varied. Perhaps the most widespread chemicals in surface sources of drinking water are a poorly characterized group of natural substances referred to as humic and fulvic acids. It is well established that chlorine, ozone and chlorine dioxide react with this class of chemicals to reduce color or ultraviolet absorbance present in many surface sources of drinking water. In particular, reactions of these chemicals with chlorine have been studied extensively (*13*). It is clear that they can serve as a source of trihalomethanes (*27*) and it is known that many other reaction by-products are formed which are yet to be identified. It is important to realize that these reactions include oxidative processes as well as chlorine substitution reactions.

From the use of the rather gross parameter TOX, a general case can be made that reaction of chemicals in drinking water results in the formation of both trihalomethanes and non-trihalomethane TOX. Choosing conditions which maximize chlorine substitution reactions ($pH < 7$) we have demonstrated virtually equivalent levels of chlorine substitution in commercially obtainable humic acids and humic and fulvic material isolated from surface water sources.

In a broad sense there is also a similarity in the level and type of mutagenic activity produced by chlorination of commercially available humic acid and comparable material isolated from surface water. In no case were any of these materials mutagenic without being chlorinated. Virtually identical specific activities of directly effective mutagens were observed in Salmonella strain TA 100 in all cases following treatment with chlorine. Incorporation of Aroclor 1254-induced rat liver microsomes reduces the mutagenic activity. These observations are consistent with the types of activity which are observed upon chlorination of water (*21–23*). Comparisons of mutagenic activity in Salmonella strain TA 98 was more variable. Fulvic acids isolated from water gave rise to nondetectable to low mutagenic activity in TA 98 following treatment with chlorine. Chlorinated humic acids derived from the same waters gave the highest specific activities in TA 98. Commercially obtained humic acid upon chlorine treatment produced the median specific mutagenic activity in TA 98.

On the basis of these results it would appear reasonable to utilize commercially available humic acids as models for studying hazards associated with disinfection by-products. Recovery of sufficient material from surface sources to perform life time carcinogenesis bioassays would be prohibitively expensive. However, this is the only method acceptable for estimating the magnitude of risks to human populations. Consequently, the use of such a model system seems essential to provide guidance to the study of disinfectant reaction products at ambient concentrations. The results of these studies can form the basis for identifying important disinfectant by-products by providing clues to how these materials might be isolated from drinking water. Obviously a substantial effort will be necessary to compare the chemistry of disinfection products produced at the high concentrations used here and the much lower concentrations involved in drinking water disinfection. Understanding of the chemistry involved will form the basis of determining how directly experiments conducted with commercially available humic acids can be applied to estimating risks arising from disinfection by-products. Even if it is not possible to make a direct comparison at the very least the use of the commercially available material should allow the design of methods for recovering and detecting the active chemicals from actual drinking waters. This in itself will be a major contribution to arriving at estimates of risk posed by disinfection by-products.

In summary, it seems much too early to decide the relative hazards associated with alternative disinfectants. Of all the issues involved the question of disinfection reaction by-products is perhaps the most difficult. Depending upon the variety of water characteristics which exist in the real world, production of by-products might be quite diverse. At alkaline pH, considerable portions of the organic chlorine substitution appears to be in the trihalomethanes (*27*). At lower pH, such as those used in the present study, much more of the organic chlorine substitution is in the non-THM fraction of organically bound chlorine. It is only after a body of data has been established that has enough depth of information to allow generalizations to be made confidently can the problem of disinfection by-products be considered solved. Therefore, the greatest need is to provide the basic core of information that can be modified systematically and logically to fit local conditions. The development of a basic experimental model and a means of field validating the results and predictions of the model under a variety of circumstances seems to be an essential step in developing a workable solution to the problem.

We would like to express our appreciation to the staff of Dr. O. Thomas Love of the Drinking Water Research Division of the Municipal Environmental Research Laboratory including Richard J. Miltner, Kenneth L. Kropp and Robert S. Canter for operating the pilot plant for generating alternate disinfectant by-products. Additionally, we would like to thank J. Keith Carswell, Alan Stevens, Bradford L. Smith and Kim R. Fox for their analytical assistance. Dr. Robert Lingg, William Kaylor and Frank Mink contributed to the preparation and analysis of chlorinated humic materials. The technical assistance of Dave Cmehil, John W. Glass, Katrina Baker and Barbara Cameron was responsible for the completion of the biological testing that was performed. Finally, we would like to thank Ms. Elisabeth Winter and Ms. Nancy Koopman for their assistance in the preparation of this manuscript.

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Environmental Health Perspectives
Vol. 46, pp. 229-241, 1982

Session Summaries and Discussion*

Session Summaries

J. A. COTRUVO: In the course of this conference we have had sessions devoted to the toxicology, mechanisms of action, chemistry, epidemiology, and clinical experience associated with drinking water disinfectants. Each of the session chairpersons will, in turn, summarize the discussions in each group.

D. COURI (Mechanisms): I will limit my remarks to the session which dealt with direct effects of disinfectants in studies involving cats, rats, goats, monkeys, and man. There appears to be a common toxicity which emerges among these species which is referable to the oxidative capacities of the Cl compound disinfectants. These oxidative agents produce effects which ultimately impair red blood cell functions. This was demonstrated in the chemical, morphological, and physiological data. For example, the decrease in red blood cell levels of intermediates such as the decrease in red blood cell glutathione produced hemolysis and morphological alterations of red cells in each of the species mentioned (except man). There are at least subtle hints that the erythrocytic function in man may in fact be under some circumstances and treatments vulnerable in the same direction. It is important to emphasize that definitive effects occurred at very high doses, much higher than we would expect to see from water disinfection. Few, if any effects were observed at the low doses, i.e., at 1–5 or 10 parts per million, which we would expect to be used in water treatment plants. It is likely that a unifying hypothesis on the mechanism of action could be derived from the original reports of Heffernan and others at the USEPA (HERL) laboratory, where it was shown that very high levels of hydrogen peroxide could be generated in the red cell after treatment of animals with chlorite. It is well known that hydrogen peroxide produces damage to cellular elements. However, the biological system has the capability of compensating for these oxidative results. Therefore, the serious effects seem to be reversible, and may be of a transient nature.

Ammar discussed preliminary studies on embryotoxicity. Certainly at high doses maternal deaths occurred after treatment by the intraperitoneal route, but the administration of high doses of disinfectant in the drinking water also produced some effects on the embryo and the fetus. Bercz has shown in the monkey a previously unreported effect of chlorine dioxide or its metabolites on thyroid function at a dose of approximately nine milligrams per kilogram, which may prove to be the most significant toxicity. This effect deserves further attention.

Calabrese's discussion concerned two mouse strains, one having sufficient, the other deficient glucose-6-phosphate dehydrogenase activity. There were no major differences in the sensitivity of the two strains. The application of the results to human glucose-6-phosphate dehydrogenase-deficient individuals is not clear, because the deficient mice still possessed a much higher activity than that observed in humans deficient in this enzyme. I think that Calabrese's attempt to use the goat for another species has drastic limitations, inasmuch as the goat's carbohydrate metabolism in the red blood cells is entirely different from that of other mammals. Again this needs careful consideration in terms of removing such confounding factors before applying the results to man.

Although it is difficult to arrive at a safe level for humans with any great degree of certainty, I think based upon the studies reported here that somewhere below one part per million chlorine dioxide might be acceptable and about five parts is perhaps a ceiling for total Cl compound disinfectant in drinking water.

M. ROGUL AND C. SONICH (Epidemiology): The first studies of chlorination and cancer in humans examined the relationship between general measures of water quality and cancer mortality rates. The purposes of these studies were not to establish causality or to derive risk estimates that could be used as the basis for drinking water regulations, but were exploratory measures in epidemiology that were used to identify suspect cancer endpoints and associate water types that should be investigated with more definitive epidemiological methods.

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The epidemiology studies that we have heard during the conference use the information of these earlier studies as the basis for their designs. That is, these studies were designed to both test the hypotheses that were generated during the earlier studies and to further explore the new ideas of cancer risks associated with drinking water contaminants. Contrary to the original exploratory studies, this generation of studies is based on newly collected information concerning cancer disease rates and water quality.

As would be expected in the progression of scientific research, the methodology of these studies was markedly improved over the original studies and was certainly more sophisticated. Two of the five studies that we have heard during this conference have used cancer incidence, and all of the studies have collected and examined much more information concerning extraneous factors such as the potential confounders: occupation, resident stability, smoking—things that might bias the results. Much more extensive information has also been collected concerning drinking water exposures. Thus, the results of these studies significantly add to our knowledge concerning the potential cancer risks of chlorination. However, since these studies still must be considered as first generation studies, they do leave a number of unanswered questions, as might be predicted.

Rather than being discouraged with discrepancies in these studies, we should be encouraged by the similarities; we should note them and exploit them to the fullest extent. The overall trend is that the risk of gastrointestinal cancers such as colon or rectal cancers is increased by exposure to chlorinated water. These similar findings should be further examined in light of the different methodologies, exposures and diagnostic criteria, in an effort to explain the differences. Hence, we should address and recognize the challenges that are involved with the exploration of low level cancer risks in the general population.

The purpose of our roundtable discussion was to determine the role of epidemiology in future assessment of the health risks associated with drinking water chlorination. We discussed and critiqued past studies in an effort to focus on specific questions indicating future research needs. If any conclusion was reached by the group, it was that based upon the epidemiology studies completed thus far, there is an association between chlorination of water and cancer.

The discussion was enthusiastic and positive in regard to past, present, and future research; pointing out deficiencies yet noting that in only seven years of studying this problem we have come quite far and are moving in the right direction. (For example, the water hardness/coronary heart disease issue has yet to come this far.) The problem is complex and must be approached in steps with attack on one objective at a time. While it is difficult to summarize such a spirited discussion, we will present a few of the main points which illustrate the consensus.

It is recognized that a causal relationship between chlorination of drinking water and cancer has not been established. But causality should not be expected at this point. The purpose of the phase I (first generation) studies was to add supporting evidence for the existing hypotheses. These studies were not intended to answer all questions but were designed as screening studies to determine which cancer site(s) is important.

The unifying thread between these studies is that drinking chlorinated water puts some stress on the human body that results in chronic disease. The studies most strongly indicate rectal cancer, but colon and bladder cancers have also been indicated.

Discussion focused on examining these studies to define the refinements necessary in designing future studies to answer additional questions (both within and outside of the field of epidemiology). The refinement predominantly discussed deals with exposure assessment. Categorization of chlorinated vs. unchlorinated or surface vs. ground does not provide sufficient information. More detailed and specific monitoring data are needed to include a variety of measurements necessary to more completely characterize water supplies. Mixtures of exposures will vary with source regardless of whether the source is chlorinated or unchlorinated, surface or ground. In this situation, it is necessary to better understand the chemistry of chlorination and its reactive by-products.

In addition, we also need to better understand the biology of chlorination, i.e., the direct effects of oxidant chemicals on the host. The biological modifications and adaptations associated with water chlorination need to be defined. Perhaps study populations should be stratified biologically, although the feasibility of doing so might be questioned. Finally, a better understanding of cancer, especially colon cancer, would contribute to efficient study design. Is there truly a difference in ascending, descending, and transverse colon cancers as related to drinking water chlorination?

Such questions outside of the field of epidemiology must be considered, but questions also arise within the field as well. Misclassification is often a problem in epidemiology studies. This is especially true when examining death certificates as done in the first generation studies. However, death certificates have been found to be 85% accurate when noting cancer as the leading cause of death. Nevertheless, the second generation studies are looking for associations with cancer incidence by using tumor registries. Since the cases will be living, more accurate cancer and exposure data can be obtained.

It has also been pointed out that the epidemiology studies arrive at low relative risks such that their importance is questioned along with any conclusions derived from them. However, although the strength of the association is weak and the risk to an individual is low, one must look at the number of people potentially affected by the disease. Thus, a small increase in risk can have a profound effect in terms of the number of lives at risk when the background rate is high, as is the case for colon cancer. The attributable risk is an important measure to consider in public health decisions.

In short, our discussion enabled us to identify important issues to consider in designing the next generation of studies, the analytical study. The first generation studies served an important function in generating questions for the epidemiologist as well as the chemist, biologist, toxicologist or any scientist interested in this issue. Perhaps one member of our group summed it up best by saying that he could salute the flag of chlorination (as a cancer risk factor) as long as he did not have to pledge allegiance for the rest of his life. We are moving in a general direction based upon results of the first generation studies and the questions generated from them. The results of the second generation studies along with biological, toxicological and chemical data will determine our next move, but for now, the emphasis should be placed on obtaining more and better monitoring data.

F. Kopfler (Chemistry): The session began with a study presented by Carlson in which he was working with a known class of compounds. After treatment with chlorine he was able to identify many of the products and, account for almost 100% of the products formed. In this model system of reacting PAHs with chlorine at different pHs, it was found that at the lower pHs chlorination and oxidation reactions occurred and at a higher pH, on the other side of the p*K* values, oxidation predominated. He identified most of the products and found really only one compound that showed some interesting biological activity—more activity than the parent compound. That was the 1-methyl-4-chloronaphthalene. In addition in to the pH the chloride ion concentration is also very important, as it should be since chloride ion takes part in the equilibrium resulting when chlorine dissociates in water.

Completely different reactions occurred when the polynuclear aromatic hydrocarbons were in true solution as opposed to being in particulate form. This was borne out in some of the other talks when we discussed reactions of some of the humic substances. Such substances tend to exist anywhere from true solutions to colloidal to particulates in nature, depending on pH and perhaps other conditions.

Stevens pointed out that the aqueous chemistry and reactions of chlorine dioxide with a lot of model compounds has been well studied and reported. He reported on what most of us here were really interested in, those reaction products produced from uncharacterized substrates found in drinking water.

The work reported by Johnson supported Carlson's finding that at lower pHs more chlorination products are observed and at higher pHs he obtained oxygenation products along with the incorporation of a lessor amount chlorine. Haloform production is, however, increased at higher pH.

Stevens showed that the reaction of chlorine dioxide at high pH results in less chlorine incorporation into the organic substrate than occurs at the more acid pHs. Nevertheless under the same conditions, chlorine dioxide results in the introduction of less chlorine into the organic compounds and in all cases, far less production of trihalomethanes.

The presentations of Johnson and Watts point out that, really to solve some of these problems, newer analytical techniques are going to be required. We discussed double focusing high resolution mass spectrometry, field desorption mass spectrometry and several others, including negative chemical ionization. Further development of analytical techniques is essential.

Although many reaction products can be identified, as Glaze pointed out in his talk, the reaction mixture still contains high molecular weight, very polar material that does not appear to have changed that much even though a large number of reaction products have been identified. This background of the high molecular weight materials does not particularly change with ozonation. They're also still there after chlorination. Dr. Johnson pointed out that the myriad of products identified in the studies at North Carolina account for perhaps 8 to 14% of the original carbon, and the same thing with chlorine dioxide. We see many products, but the TOC (the total organic carbon) has not changed appreciably.

The one study reported on chloramines is really just in its beginning stages. We don't know much about even the simple inorganic mono- and dichloramines. We have a lot to learn from just studying the kinetics and dissociation of the disinfectants; but most particularly the chloramines. This basic information is needed before we can really come close to predicting what any of our products of alternate disinfection are going to be.

J. F. **Borzelleca** (Toxicology): In our session we addressed the potential adverse health effects of the trihalomethanes. We conducted two series of studies: those with mice and those with the rats.

The long-term studies in female mice (B6C3F1) that Jones described are lifetime studies that attempt to evaluate the carcinogenic potential. This study is in progress; it's not complete. It is a well-designed, and well-executed study. We are eagerly awaiting the results. In our program in Richmond, we are evaluating the general toxicity of these materials. We have determined acute oral LD_{50} values and 14- and 90-day subchronic exposures. The materials were administered by gavage rather than in drinking water to permit exposure to high levels (their solubility is limited).

Our subchronic studies reconfirmed the liver as the target organ for the trihalomethanes. An effect on the spleen was also noted. Subchronically, chloroform appears to be the most toxic of the four halomethanes that were evaluated, bromoform the least. A degree of hyporesponsiveness or "tolerance development" was observed in several immunological endpoints, the humoral immunity and cell mediated immunity, and the functional activity of the RES. The spleen weight was decreased and there was a decrease in colony-forming cells. These effects were seen only at 14 days and not at 90 days, suggesting hyporesponsiveness or tolerance development.

The trihalomethanes administered orally did not affect DNA synthesis. Effects were seen only when the material was administered intratesticularly to the mice. This reflects the effectiveness of the blood–testicular barrier. Multigeneration reproduction studies were conducted using high doses. There were no adverse effects on any of the usual reproductive parameters through three generations.

There were few significant behavioral effects using a number of systems (including some developed in our laboratories). Nothing suggests a progressive neuropathy or neurotoxicity. Using the taste aversion paradigm, the effect of chloroform was noted at about 30 mg. A progressive hyporesponsiveness or tolerance development to the trihalomethanes was also observed.

It was reported that chloroform stimulated both ornithine decarboxylase activity and the RNA polymerase 1 activity in the mouse. These are involved in the early stages of the carcinogenic response. Jones' study with Osborne-Mendel rats is not complete. Levels up to 1800 ppm in drinking water were used. This study should resolve the issue of carcinogenicity in rodents. The data are not available yet. There were early deaths in the mouse because they were not accepting the drinking water. There were very few deaths in the rats. Excellent survival among rats at the high levels of chloroform were noted.

Data presented suggests that chloroform is not an initiator but is probably a promoter. It was reported that hepatic ornithine decarboxylase activity in the rat was stimulated by chloroform. This stimulation of ODC decreased with time. ODC activity in the kidney was depressed. Apparent tolerance to chloroform was noted.

Based on detailed toxicological investigations involving doses that were orders of magnitude greater than maximum anticipated human daily consumption, and which were administered acutely or subchronically, it can be concluded that the trihalomethanes are not highly toxic. The hyporesponsiveness or tolerance development to the effects of THM were demonstrated in a number of systems including the immune system and behavior, and the synthesis of systems including the immune system and behavior, and the synthesis of hepatic ornithene decarboxylase in both the mouse and the rat. The THMs stimulate the synthesis of hepatic ornithine decarboxylase RNA polymerase and RNA polymerase activity. Chloroform appears to be a promoter and not an initiator of carcinogenesis. Many of the effects reported were seen only at high doses.

R. J. **Bull** (Biological Test Systems): I think there are a few things to which we can call attention. The first is that there is some reason to be concerned about the so-called nonvolatile portion of the organic material in drinking water. It is now clear that treatment with certain disinfectants increases the level of certain types of biologically active molecules. This was most convincingly demonstrated on the papers of Zoeteman et al. and Kool et al. The mutagenic activity observed is not going to be attributable to the trihalomethanes. One reason is the concentration technique employed would not particularly recover the trihalomethanes. A second reason is that the trihalomethanes have little or no activity in the test systems used. Third, the activity of the trihalomethanes would not be the direct acting type indicated in the results. So, there are a variety of biologically active compounds that are generated in this nonvolatile fraction by chlorination that can be identified as potential problems. That is a long way from saying that the risks due to those materials are high, low or intermediate. One of the major conclusions of this conference will have to be that toxicologists, chemists, and epidemiologists are going to have to focus on the overall problem of disinfectant by-products. We should not get completely entranced by the idea that correlations of health effects with chlorination of drinking waters is a simple one involving only trihalomethane formation.

There were several other things that I do not remember being specifically articulated but can be derived from the presentations. In my own presentation, I failed to call attention to one parallel between the toxicological and epidemiological results. Isaacson reported that there was a possible relationship between agricultural runoff and the incidence of cancer in the epidemiological studies. Ottumwa, Iowa was a city selected for study in our work for that reason in our five-city study. It was the only city to give rise to significant increases in tumor incidence.

So far I have been speaking about problems associated with chlorination. Here we are really just beginning to understand the identity of by-products and tend to focus our attention on individual products. However, we must also begin to recognize the more general problem; that the use of strong oxidants for purposes of disinfection alters the chemical nature of the materials that are natural background in any surface water. There is some concern with synthetic organic chemicals, but it seems that in surface waters much of our problem with chemicals in drinking water now involve and will continue to involve disinfection reaction products with natural background material. This is the source of the trihalomethanes, and it seems to be the source of a sizable portion of the so-called TOX (total organic halogen) in drinking waters.

One of the problems that must be addressed when taking a bioassay approach to the field is that these methodologies are not chemically specific. They are specific for a particular type of biological activity. There are variations in and among source waters and in the same source water at different times of the year. There seem to be differences in the amounts of total organic halogen produced over the course of a year. With surface water sources like the Ohio River, a few hours or days time can result in substantial changes in the synthetic chemical composition of the water.

Variability in biological responses are also encountered, so it becomes important to avoid placing a great deal of value to the results of one experiment. I think that Zoeteman and Kool indicated there was about a threefold variation in the level of mutagenic activity they were seeing in the Rhine and Meuse in the course of a year. Bioassay results are likely to give a misleading picture of this variability because they do not distinguish between different chemicals, but integrate levels of a particular biological activity potentially caused by a large number of chemicals. Therefore, bioassay does not take the place of chemical analyses. Consequently, we must be as systematic as possible from both the biological and chemical standpoints in trying to characterize what's happening with processes such as disinfection. Efforts should be directed toward developing a fundamental understanding of the problem. Once the chemistry is better understood and model chemicals are examined for toxicological properties, we could then look at actual drinking waters with better tools and minimize the methodologic problems that invariably arise with the use of any sample concentration methodology applied to organic chemicals in drinking water.

Chlorinated phenols are by-products of chlorination which have been repeatedly identified in drinking water. Koller's presentation dealt with chlorinated phenols. 2-Chlorophenol and 2,4-dichlorophenol have been poorly characterized from a toxicologic standpoint. The 2,4,6-trichlorophenol has been shown carcinogenic in the NCI bioassay program. It is significant to note that Koller is beginning to observe some toxicological effects of 2-chlorophenol, indicating that there are individual chemicals within that mixture that possess some of the activities that we're concerned with. It is unlikely, however, that a chlorinated phenol would account for the direct-acting mutagenic effects; my guess would be those compounds would be indirect-acting if they are active at all.

The last talk dealt a development that could add a great deal of specificity to epidemiologic investigations—that is binding of carcinogenic and mutagenic chemicals to macromolecules. Utilizing this property provides a potentially elegant means of establishing relative contributions of active chemicals like carcinogens may have to human disease processes. I believe that if someone would get very clever with the approach of using hemoglobin alkylation as a dosimeter it would be possible to begin to weigh a variety of exposure to chemicals in an integrated way. Using this analytical tool, it would be possible to determine the relative level of exposure to a multitude of chemicals. Dr. Pereira's group has looked at the *in vivo* binding of 16 chemical carcinogens to hemoglobin. These chemicals included indirect, as well as direct acting chemicals. Therefore, the binding of carcinogens to hemoglobin is a fairly general phenomenon that could be extremely useful in sorting out confounding exposures in epidemiological investigations.

L. J. McCabe (Clinical Studies): We discussed two approaches, the epidemiological and clinical. One of the epidemiological studies was the more classical type where you dragged up available data from a great many years in the past such as Tuthill's and Moore's study, and the other was the more elaborate clinical chemistry used in the study reported by Miday. These are examples of following up on an existing practice of chlorine dioxide used for disinfection and concluding that nothing life threatening seemed to be occurring, and that these practices probably could continue to be employed. Tuthill's study certainly would indicate that if you introduced this new practice of chlorine dioxide in a community, you might want to set up some kind of health surveillance to keep track of those few things that might show shifts. He did indicate that there were some population shifts using it over this short time period and alluded to the fact that these population means could continue to shift if it was carried out over a longer period of time. So those things might be incorporated into a new change if some community was going to follow through on using chlorine dioxide.

It's possible that these techniques of Tuthill and Miday could also or should very appropriately be used on a community that's been using chloramines, because most of the studies that were discussed earlier were pointing a finger at what happens when you use chlorination. So we do have some possibility of using the epidemiological approach on chloramines as was done with the chlorine dioxide.

The final paper was presented by Reitz, a representative from Dow. I don't know whether it was apparent to those of you from the water utilities, but the two approaches being used would really make quite a difference, I think, to you. The trihalomethane limits using the epigenetic approach would be something like 100 μg/l.; if you used a genetic approach, it would probably be 2 μg/l. We have a factor of 50 apparently in between those two approaches to what might be considered as trihalomethane regulation.

J. A. Cotruvo: I think those summaries were excellent in focusing the essence of the last few days of discussion. The title of this session is in part at least, discussion of regulatory issues and I'd like to just mention a few words in that direction before we open the discussion to the floor.

The decisions that a regulator makes perhaps are made with a somewhat different perspective and with somewhat different motives than some of the motives that influence the work that you have heard. A regulator, of course, includes all of those factors in the decision process, the toxicology and the epidemiology that exists, and any other indications of risk that can be computed. At the same time the decision context is not necessarily the obvious one. It's something that is determined by what the Congress has written into the law that tells one how to treat the information that's available and tells one what kinds of factors have to be taken into consideration. In addition to all the toxicology and the risk part of the equation one also considers the burden of proof, the quality of evidence that exists, and the cost and the practicalities of whatever the control options might be.

As you know, some decisions have already been made. In 1979 there was a regulation established for trihalomethanes as indicative of contamination of drinking water resulting from chlorination. The question that we ask ourselves—and that I would hope to get some more guidance from this meeting — is, was that a sufficient regulation by establishing a maximum contaminant level for that limited group of substances? Did that in fact change the national risk picture substantially? We are assuming that in the United States now as more and more communities take steps to reduce trihalomethanes, drinking water risk factors should have been reduced to some degree and hopefully they'll be reduced further in the future. The question is, do the data indicate that it's necessary to take more drastic action or more costly action in the future?

There were also some peripheral decisions made in the course of that regulation and in an advisory sense relating to the alternate disinfections such as chlorine dioxide. The suggestion was made that the inorganic residuals of chlorine dioxide derived by-products should not exceed one-half of a milligram per liter in cases where it was used. There were also suggestions made about the appropriateness of using ozone in certain situations where there is high organic water and where the probabilities would be that difficulties and quality problems would be caused as a result of ozone. Obviously, as a result of that regulation there is a very substantial increase in the use of chloramines, at least as distribution system disinfectants. There are a great number of unanswered questions as to whether the fallout that occurred from that regulatory decision in fact results in an increased protection of public health or possible substitution of one set of risks with another set of risks.

So, my conclusion is that clearly we have a very confusing situation here. I'm not sure that we are ready to answer very many of those questions that I just asked. But in the context that we all operate—which is one of various kinds of pressures, or at least motivations, some of them being limited by financial considerations—just how much information is necessary before one can make a certain decision in this regulatory context? The pursuit of knowledge for the sake of knowledge is, of course, an admirable direction to take. The question is, in which areas should the limited resources that we have be expended? Where is the greatest potential for pay off? Will increasingly sophisticated and expensive study in a particular area in fact lead to some different set of conclusions or ome different set of decisions? If the relative ris of exposure to disinfection by-products, and for ex mple, the relative cancer risk turns out to be 1.3 ins ead of 1.2, does that lead to a particularly dif rent set of decisions? If the relative risk turned ou to be 10 instead of 2, that might lead to some ve different decisions!

o the question is, where do we draw the line; wh re do we direct the limited resources that we ha e; where is the best pay off going to be, and to wh t degree should we divide that resource pie by in estigating this aspect of drinking water quality ve sus other aspects of drinking water quality and ris s associated with them. These include problems su h as cardiovascular disease risks, trace metal co tamination problems due to corrosion, etc., and bio ogical problems which are not resolved. We mu st still consider water quality problems from bio logical contaminants. Keep in mind that in those cases we can "count the bodies" whereas in this case of disinfection by-products risk we can't, and so there are many difficult decisions to be made.

So there are a lot of difficult choices to be made and we have to make them, and unfortunately there are restrictions. So we have to decide where to put the resources and where the best payoff of human health protection will be, even though there may be some areas of interest which will not be pursued as a result. So with that then, I will open the floor for questions.

Discussion

T. D. JOHNSON (Univ. N.C., Chapel Hill): I'd like to focus on a couple of things that seem to me to need balancing in looking at the problems that we face. Most of the meeting was focused on the issues of rihalomethanes because we found trihalomethanes we re easy to measure and we therefore hung our ha very much on trihalomethane levels. This has gi en us a number of biases. One question that ne ds to be asked particularly is, are the chlorinatic n by-products from natural aquatic humic materi s, which are major by-products from woody tis ue the problem, or are the chlorination bypr ducts from biological by-products the problem?

think Bull's comment that the city which sh wed the best correlation was the agriculture ru off city is particularly germaine in this question, ar I think that the question needs to be asked be ause we have focused so much of our effort on th woody tissue chlorination by-product because of th nice, easily measured chloroform. In particular, th re was one paper which began to address this question of biological by-products: Scully's paper, which dealt with the natural organics issue. It seems to me that the organic nitrogen compounds or other biological by-products as they are chlorinated are of major concern and I see very little of that type of work being done. Although the one study I am familiar with, the 5-chlorouracil I mentioned before, gave in a small rat study a negative teratogenic response, the fact is that 5-chlorouracil was taken up to the extent of 3 per genome in DNA, and that in itself I think is a rational basis for concern. So I personally would like to see more of this kind of effort.

The other kind of effort I think that needs to be mounted is the need to couple the toxicological, epidemiological and chemical studies together. As a chemist, although I'm surrounded by biologists in my department, I'm bothered by the fact that I don't know when I do my separation processes, which are necessary in order for me to get any meaningful results, that the fractions that I am actually spending my time on are the interesting ones. It's clear to me, I don't know how it is to the rest of you, that chlorination by-products are a concern and the problem to me is, which ones can we focus on? I think this coupling of the chemical studies with the necessary separations that we have to do as chemists with Ames tests and with toxicological tests are a needed factor. I would like to see more joint studies.

J. A. COTRUVO (U.S. EPA, Washington, D.C.): I think you made a good point, which I would like to emphasize and that's in the matter of the biological components in the water. Traditionally, public water systems get very excited when they have algal blooms in their waters because this adds a bad taste and causes a lot of consumer complaints and so they will do anything that they can to avoid that situation, including moving to other disinfectants that don't provide the taste. Well, what has not been perceived to date has been, in fact, the reason for that taste is a very substantial increase in the chemical loading of that water, and that many of the measures that are being taken to ameliorate the taste in fact further increase the chemical complexity of the soup that's been produced. Addition of larger amounts of chlorine or other oxidants may, in fact, compound the problem. So if there are risks from organic chemicals and drinking water it's reasonable to assume that during those periods of time when algal blooms are occurring and tastes are being added, additional chlorine is being added and the risks could be substantially increased. So it may be that a different perspective needs to be placed in the minds of water plant operators as to how to respond to those kind of situations.

R. J. BULL (U.S. EPA, Cincinnati): I would like to make one comment about Johnson's statements here: I think the biological testing part of the disinfection by-products product business needs to be focused. In the case of potable wastewater reuse, the circumstance which you are talking about with the 5-chlorouracil is even more likely a problem. Just a very quick look at some data that have been generated around with some of the reuse plants or potential reuse projects around the country, one thing that stands out is the percentage of the organic material that's in the form of organic nitrogen. It is certainly higher in that circumstance than it is in most places where we draw our water from streams where the nitrogen is probably captured and utilized by microorganisms along the way.

K. P. CANTOR (Natl. Cancer Inst., Bethesda): One or two remarks on some of the items that have been brought up for discussion so far and partially in response to the question as to whether trihalomethane regulation might have public health benefits. There's one crucial piece of data which involves environmental measurements that we do not now have which would be very important to have—that is what is the association between levels of trihalomethanes and levels of the genotoxic substances that are below the surface of the water that are the rest of the iceberg. I think the answer to that question would be more readily forthcoming. And I see that as a very strong research need that has come to my mind anyway, as a result of the discussion in this conference.

The remark was disturbing in another sense too. I think this is a time for toxicologists and epidemiologists to learn a great deal from each other and can contribute to the direction that research is taking in the other's field.

J. STARA (U.S. EPA, Cincinnati): You know we have investigated water for many years, and I remember some years ago when they found some 700 compounds in Cincinnati's drinking water. I am surprised that you say that we do not know what compounds besides halomethanes are in chlorinated water or as a by-products of chlorination. And my specific question is, did Koller say that trichlorophenol and pentachlorophenol have some relationship to carcinogenesis?

J. A. COTRUVO: On the statement about not knowing what the substances are in drinking water, I think that one of the major points of the chemistry part of this meeting is that we know some of the compounds that are in drinking water. We can identify several hundred or several thousand, but when you add that all together, you still account for only perhaps 10 or 15% of the total mass of chemicals in water; thus one of the questions is, how far should that be pursued? Will we really be able to make decisions on the basis of identifying additional substances, or is there some other approach that will lead us to a conclusion more quickly.

UNKNOWN: You said that the THM serves as an indicator, but don't you think there will be utilities treating just for the THMs and ignoring the total organic chlorine, I mean the way it's set up?

J. A. COTRUVO: The assumption is that it won't be possible to treat only for the THMs. In the course of reducing the THMs, as a natural consequence many other substances will be reduced too, unless the method of reduction is some very selective process. Offhand, I don't know what that selective process would be. Some people might say aeration, but even that removes some number of substances in addition to the THMs as well as chemically transforms some of the substances that are there, so I don't think there are any really unique processes that would selectively remove THMs and not touch the rest. So with the information that was available in 1979, that was the most reasonable course to take, fully conscious of the fact that it was intended as a surrogate for all of the aggregate of the substances that are in there.

R. J. BULL: I think that has at least partially answered the question about the identification of chemicals. A lot of them have been identified, but I think everyone that is here recognizes the fact that one thing that has been established in this meeting is that there is biological activity associated with those parts of the material isolated from water which are completely separable from those chemicals which are identifiable chemically. So I think in some ways that it looks like the submerged part of the iceberg is a larger problem than that involving known chemicals. The answer to Stara's question about Koller's paper is that he presented preliminary data, which indicated a trend in the direction of a cocarcinogenic effect with both 2-chlorophenol and pentachlorophenol.

J. A. COTRUVO: Just for the record, trichlorophenol came up active in the NCI bioassays last year.

J. STARA: My only comment is about these many other chemicals. It's true that we know almost nothing about some of them but we know something about many of them. We know we have some toxicologic or in some cases epidemiologic data on other chemicals besides trichloromethane.

R. J. BULL: Yes, we do. The biggest problem in that respect, I think, is that the amounts are so small once you get past the trihalomethanes so as to be of very questionable significance on an individual basis. I think that's the thing that we're trying to deal with.

J. A. COTRUVO: Usually well below parts per billion concentrations. The number of chemicals identified in drinking water is in the thousands now. There is such a small amount of each there and it's so variable. Data will show something is in the Ohio water today, but it's not there tomorrow. It will be very hard, I think, to deal with that variability. These disinfection by-products result from the reaction of the disinfectant with these natural materials which are always there and probably fairly similar in all waters. It is hoped that we're going to be dealing with the majority of the organic material in water when we deal with these reaction products of the humic substances with the disinfectant or amines from biological origins. This is a situation that is peculiar to drinking water and is probably specific to drinking water as opposed to other problems we see with environmental chemicals. This is something which is pretty much a universal problem in drinking water, and I think it's important to address that one for that reason if for no other.

Unknown: Given the data on the changes in the intestinal flora in response to the possible risk factors from large bowel cancer, e.g., decreases of bacterial diversity in individuals of high fat diet, has any work been done or planned to examine the effects of disinfectants or their by-products on intestinal flora? And I was very glad to hear that Rogul introduced himself as a microbiologist.

M. Rogul (U.S. EPA, Washington, D.C.): I think it is a very complex question. I did have some pertinent experience, because at one time I worked as a veterinary microbiologist and the use of the mouse posed problem which is germane to this question. It was found in the early studies on radiation that many mice which were being raised commercially harbored *Pseudomonas aeruginosa*. When these mice were irradiated, their immunological barriers became attenuated. The pseudomonads proliferated, and thereby caused disease and deaths which ruined many experimental projects. It was later found that the addition of chlorine in the form of sodium hypochlorite or hydrochloric acid in mouse colony drinking water could effectively eradicate *P. aeruginosa* from these mice. To my knowledge this is the way that all commercially produced mice are reared. I think most researchers are probably dealing with mice that have been drinking highly chlorinated water for numerous generations, and it has a profound effect on the intestinal flora of the mice. We found that this type of chlorine treatment also seemed to eradicate pseudomonads from the nasal turbinates and middle ears of mice. In response to the last part of your question, I don't know of any research ongoing or planned at the EPA which would answer your question.

In addition, since most mice are raised on chlorinated water, and I understand that chlorine can be metabolized *in vivo* into trihalomethanes, I think it would be prudent to interpret experimental toxicology data with this in mind.

C. Mang (Buffalo Power and Water Admin., Saskatchewan, Canada): I have two questions on algal toxins, both relating to health effects research that either has been done or is under way on the subject of health effects of chlorination of blue-green algae or of algal toxins and their metabolites. Is anyone aware of studies along those lines? There are studies that I'm familiar with on chlorination of algae with the intent of demonstrating that there are by-products including trihalomethanes. But [is anything known] on the toxicity?

J. A. Cotruvo: If I recall correctly, one of the requests in the research strategy for this year does include studies of this sort but I don't know of anything that's underway right now. I guess we haven't heard of anything.

The next written question submitted is what epidemiological studies have been carried out relating to prolonged ingestion of carbon tetrachloride in drinking water and the possible effects of carbon tetrachloride on hepatic carcinogenesis.

C. Sonich (U.S. EPA, Cincinnati): When you say prolonged effects of carbon tetrachloride that's difficult to address, because carbon tetrachloride has not been routinely monitored in drinking water; so it's hard to assess effects due to prolonged exposures. We recently had the opportunity to study the effects of a carbon tetrachloride spill into the Ohio river. It was an assessment of an acute exposure to a relatively high dose. Seventy tons of carbon tetrachloride were spilled upstream from Cincinnati. We did a study looking at patients that were in the hospitals along the river during a time when we knew there were high concentrations of carbon tetrachloride in the drinking water compared to a time when we knew there was virtually no carbon tetrachloride in the drinking water. Kidney and liver function tests routinely performed on patients upon their admission into the hospital were evaluated. A dose/response relationship for creatinine, a kidney enzyme, was indicated. Controlling for disease, the number of patients with elevated creatinine levels was higher when the dose of carbon tetrachloride was higher in the drinking water. As the dose decreased downstream, so did the number of people with increased creatinine levels. This could signal an effect due to the ingestion of carbon tetrachloride or may be due to random chance. Dr. Bercz, would you like to comment on the toxicology study we designed to verify this finding?

J. P. Bercz (HERL, U.S. EPA, Cincinnati): The study tried to reproduce the actual dosages experienced during the spill. By the administration of such water to rats we tried to explain the apparent human effects. We have attempted to identify renal lesions as well as hepatic lesions in a two-week exposure which approximately equates the time of exposure in the human population. We could not detect any adverse effects on the animals. There was rather extensive workup of all the serum chemistry and hemotology parameters, and there was none that we could identify as adverse.

Unknown: Carbon tetrachloride is carcinogenic in the B6C3F1 mouse being used as a positive control in a number of the NCI bioassays of the chlorinated hydrocarbons. And when you say a high concentration in the Ohio River, it was not high compared to what a toxicologist administers to an animal to get an effect. The levels observed did not exceed a few hundred parts per billion at any water intake on the river, maybe 340 ppb or so in Huntington. Where there were larger populations at work, it was no more than 50 ppb to 100 ppb and then for just a short time, a day or so. The highest we were able to detect in just the drinking water was 280 parts per billion. You would not see much chemical changes or organ changes in any animal at these concentrations.

A. A. Stevens (U.S. EPA, Cincinnati): You made the point that no epidemiological study is very neat. I've just gone over that data with a carbon tetrachloride spill from our station in Louisville, and although we didn't have as high a level, there was a coincidental chloroform spill at the same time, of near the same magnitude and I didn't believe it either until I went over the data. But, it goes to further illustrate the point that, in that instance, assuming the carbon tetrachloride was high, not only was carbon tetrachloride high, but there was also a coincidental spill.

C. Sonich: From the available monitoring data we looked at trihalomethanes and other pollutants in the drinking water, and we saw relatively low levels at the time of the spill, so I'd be interested in looking at your data.

Unknown: This was found in the Huntington intake, too, so I don't know where the spill took place, but there was chloroform found in the raw water at that location—not nearly at the same concentration as the carbon tetrachloride, but then we may not have sampled at the right time.

J. F. Borzelleca: I would like to comment on the method of administering the trihalomethanes, using gavage versus drinking water. Gavage is used for a number of reasons. One, so that we can carefully note the dose that the animal is given. You're giving him a specific amount based on body weight or surface area and it's very carefully measured and administered. Secondly, this method is used because of physical and chemical problems with material—can't get it into solution, at the concentrations required for the test. We recognize that there are differences in the kinetics of giving it all at one time versus sustained exposure when it's in drinking water or feed. At least in our data, we don't find differences in the metabolism of the transformation. The same metabolites are formed but at somewhat different rates. Certainly, the peak blood levels are different and absorption would be faster. Most of these metabolites are absorbed quickly from the gut, so it does depend on concentration when it's administered by gavage and when it's a more sustained effect.

Some years ago, we did a study using one of the food colors, either putting it in drinking water or giving it by bolus administration. We saw effects only when administered in a bolus form and not when administered by the other two routes. There are differences using that kind of administration. The other question, also from the same individual, had to do with the effects we saw in the testes on DNA and when the material was administered intratesticularly, intraperitoneally, or by mouth—why did we not see an effect when given by mouth or why did it take so long? Fortunately for us, in both males and females, the testes or sperm is protected by a blood-testes barrier. Some materials do get through the barrier under certain conditions. The work of Dixon and Lee in North Carolina, among others demonstrates this and some of our work as well. You bypass the barrier when you administer the material intratesticularly. When it's given by mouth or intraperitoneally, you may see an effect, as we did. It took a long time, primarily because of blood flow and just inability for much of the material to get through the testes barrier. So, in fact, we are protected. When we look at the presence of materials in the serving, for example, just about anything you take by mouth would be present in the extracellular fluid late, but in very small levels. When you do distribution studies, as we did with kepone, for example, you find very little crossing the testes barrier. But if you give the materials intratesticularly, you will find a rather marked effect on sperm development and on DNA. It's then simply a matter of a protective barrier.

I'd like to note that in the Hazelton study on chloroform utilizing gavage with 250 mg/kg/day, they did not get the increase in longevity and controls we got feeding in the drinking water, so there truly are differences in the area. Concerning this business of getting chloroform in solution in the drinking water, it just takes an awful lot of mixing. It will go, but it takes a long time.

In Britain, when they used the corn oil material and tested it against an aqueous vehicle for chloroform in mice, they got substantial differences in the tumorogenicity in the kidney. So, there was a big increase when corn oil was used.

Corn oil is not without problems itself. We have found that in both the immune work that one is familiar with and the behavioral studies, the corn oil—as pure as it appears to be—is not without effect on some of the systems.

Maltoni has done some testing with corn oil versus water with vinyl chloride. The corn oil administration produces as a great difference as when you give it by inhalation.

J. A. COTRUVO: Once we were looking into polynuclear aromatic hydrocarbon human exposure and trying to do source contribution studies. Even in the case of benzopyrene, we were finding on the order of about 50 μg/day benzopyrene in the diet and other PAHs, in addition. A substantial amount was from vegetable oil intake. I don's know how much corn oil might contribute, but that might be another confounding factor in animal studies if residual PAH levels are high in the vehicles.

UNKNOWN: What strain of mouse are you using? I think Dave showed some data that showed increased fat in the liver of the B6C3F1 mouse, and I gathered from Al's presentation that there was really nothing to be seen in your mice. I wonder if that's the strain difference.

J. F. BORZELLECA: We are using CD1–Charles River.

Two things that we've touched on—how to best spend limited resources and how to proceed that toxicologists and epidemiologists can better complement one another. I think one of the things epidemiologists have shown in the studies—and the thing we sometimes lose sight of—is the fact that only things that have shown up with any consistency have been colon and rectal cancer. I think that the question about the effect on the intestinal flora is an example of the type of experiment that could be done to take advantage of the fact that we might want to look more closely at other risk factors or what we've come to know about rectal and colon cancer, and that in using the knowledge that epidemiologists have gained in that respect in the study of those diseases, we might better allocate the funds in our research efforts.

J. M. SYMONS (U.S. EPA, Cincinnati): In spite of both your and our urging the water utilities to improve the chemical quality of their water from an overall point of view to the extent possible, most of the utilities feel that the least cost solution is the one that they will choose regardless of any peripheral effects. I wonder if you anticipate our heading towards giving any more guidance to the utilities in terms of either the control of other by-products or the use of alternate disinfectants, so that we can possibly go beyond merely the markers of THMs and on to other regulatory items that might further improve water quality?

J. A. COTRUVO: Well, I don't know. I guess what you're saying is, as a result of the conference, are there some specific conclusions that one can draw on the various items such as chlorine dioxide or ozone and that could add to either the guidance or the regulations, and should we then busy ourselves with other things besides just disinfectants and their by-products. I find myself on the matter of chlorine dioxide in about the same situation that I was when I got here. There have been a number of studies, generally with negative results, but certainly the studies themselves haven't explored all the possibilities that have to be explored before one can make a decision that could result in very widespread exposure to the population to a new agent at high levels. I would say that the studies reported here are some of the best I have seen to date. I lean very much towards studies that examine clinical effects in humans. When they can be either shown or not shown, the level of credibility on the decision is exceptionally good, and those were kinds of studies that were done, and I think that they were very successful in the areas in which they looked.

The remaining question is, are there some other areas in which they should also look, such as with young children or other parts of the population that might be susceptible to oxidant stresses. I'm somewhat reassured from the data that were presented that there do not appear to be overt toxic effects, at least among healthy individuals that were tested with those disinfectants and their degradation products. Where that leads me is to say that we should probably continue to be cautious and still strive to minimize the unnecessary introduction of any chemical entity into drinking water, and if there is a need for that substance, such as disinfectants, take pains to eliminate as many of the side effects as possible: for example, reducing dosages as much as possible, minimizing by-products as much as possible, eliminating reactants in the water that will result in increased dosages as much as possible. It seems to me to be the common sense approach.

When one is trying to deal with a problem that has such a broad spectrum of receptors—the millions of people, and broad ranges of physical states or health—just from the mathematics of it, I think that we have to assume that there's a reasonable probability that some portion of that population is going to be somewhat more susceptible to whatever the end result would be than the average person in the population. At this point in the chlorine dioxide area, I could say we are somewhat satisfied with the data that are being presented but there are still other kinds of extensions of that work that will be done before a more definitive statement can be made.

On the rest of the situation, I don't think we're ready to make any more specific judgments beyond what's already been made. I think that if we look back into the history of water treatment, there are some good lessons to be learned. Traditionally, simple solutions to complex problems are the best. Fortunately, there are such things in water treatment. The best example of that is chlorine, where, if one wanted to take a highly sophisticated look at waterborne disease transmission, one could have spent millions of dollars and decades of time identifying all the particular pathogens that might be in water at a particular time, devising analytical tests, and devising monitoring frequencies, or developing antidotes. The simple solution happened to be a very cheap chemical that pretty much across the board dealt with the pathogen problem. It may be that we're in the same spot when dealing with trace contaminants. I know that you as engineers, are proponents of this approach. If there's sufficient information to suggest the existence of the problem, i.e., contamination, instead of expending the resources that might be spent to further elucidate risk potential and to quantify it very precisely, it may be that the most direct approach is just to solve the problem rather than to further explore and identify it. In this case, as has been discussed, if we can't solve the problem, we can certainly minimize it by using the kinds of techniques that are available to water engineers to reduce the presence of extraneous substances in the water so that if there is a risk, that risk will have been considerably reduced. In fact, that's what we would have done ultimately anyway: had we explored the problem further and precisely quantified it, eventually then we would wind up with some engineering solution. So, one point of view is why not just apply the solution, anticipating the potential of the problem? I think that's still our philosophy—avoiding unnecessary contamination of drinking water and minimizing that contamination whenever it is feasible to do so.

But I think cheap is the operative word. If chlorine had been $10 a pound, back in the 1900s, how they would have tackled this problem, if they wouldn't have tried a more elaborate scheme. I think the common sense approach costs money, and we're having trouble convincing the utilities to spend that money just because the likes of you and we think that it is prudent.

I thought that was one of the things that was admirable in the presentations of Zoeteman and Kool, because their approach in The Netherlands has been to look at the situation with the best handle they had and to do everything in their power to reduce whatever hazards they were able to identify. It seems as though they are able to operate there without having the absolute definition of the degree of hazard that's involved. You saw in their presentation that they very quickly adjusted treatment conditions to lower the generation of mutagenic activity in their waters from chlorination. They changed very directly from breakpoint chlorination, but it does not quite follow that we can follow this same premise in the U.S. The country is a lot bigger. You can do a lot of things more easily in a smaller group than you can in a larger one.

Obviously, it has been shown that a wide variety of substances are introduced in drinking water as a result of disinfection processes or oxidation processes. A considerable amount of effort has concentrated on identifying those substances and has certainly shown that some of the principal ones—trihalomethanes—are ubiquitous and that there may be toxic consequences from exposure to those substances. That kind of information came primarily from animal studies at high doses. At the same time, there have been almost 30 epidemiological studies of various types that have been conducted to date with varying results, but at least some thread of relationship between variables relative to chlorination or other aspects of water quality and increased risk of cancer of certain types. A number of test systems have been devised to obtain more information on the aggregate of the chemicals in the water, given the difficulties of dealing with individual substances and the problems that one always has in identifying toxicology of individual chemicals when in fact the exposure is to a variety of chemicals simultaneously. So, the toxicology becomes especially complex.

Some very innovative work is therefore being developed in the area of concentration of water samples and test systems that can demonstrate relative effects of those concentrates as well as pure chemicals in rapid biological measurement systems. Animal surrogates for evaluating human risks have their benefits because they do allow a relatively controlled experiment. However, they have their disbenefits because they involve nonenvironmental dosage levels and of course necessitate the use of nonhumans as targets for the tests. So, those two heroic extrapolations have to be made whenever that kind of study is done. At the same time, there are some endpoints that can be investigated in humans, and studies have been conducted where actual human volunteers were exposed to various substances and a great number of endpoints were observed. Fortunately, at least in the studies reported, most of those appeared to be negative, at dosages similar to drinking water levels.

A great amount of technical work has been done and there is certainly the possibility of performing much more. The question we have to ask is which of that work should be done and where should the emphasis be placed in the future to give us the best payoff in being able to arrive at decisions that have to be made on the questions of water quality and risks associated with drinking water contamination? One of the things emphasized in this conference was the matter of trying to unite some of the different disciplines in the investigations that could emphasize the benefits of each of the disciplines, such as studies of the type done at Bethesda, Ohio, even though it was a small one. It was one where the population was studied in its natural habitat by careful clinical studies and investigations; numbers were collected on those people in an attempt to try to find any endpoints that result from that exposure.

So, given the limited data—which are in one sense limited but in another sense substantial by comparison and have pretty much been developed in the last five years—we are at the point of concluding that there may be risks from exposure to substances in drinking water and that there are ways of dealing with those substances and at least attempting to reduce those risks. So, now that we've lived through the THM era—and I think we all understand now that THM is only part of the question—we need to find out if there are ways to get through this very complex question. We must steer the research in the direction to answer specific questions. We need to decide what the question is so that we'll know it when we get to the answer. For example: which substances, which toxicological endpoints, which surrogate tests and what is their validity in representing actual human experience? So, we must ask the questions very specifically at the front end and design the research that will resolve them in the future.

I commend all of the speakers and all of the participants. I think it's been an excellent workshop and it's been a good opportunity for all of us to get together and communicate more than we ever have in the past on this subject, at least to a broader extent. All I can say is keep going; there is much to do. We need that information to make the regulatory decisions that could be very significant in terms of the health of the country.

Announcement

International Conference
Heavy Metals in the Environment
Heidelberg, 6-9 September 1983

The Fourth International Conference on Heavy Metals in the Environment will take place at the University of Heidelberg, West Germany, 6-9 September 1983, under the chairmanship of Professor German Müller of the University's Institut für Sedimentforschung.

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